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A Study of Some Physiological Processes of Corn as Affected by N-(3,4-Dichlorophenyl) Methacrylamide.

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A STUDY OF SOME PHYSIOLOGICAL PROCESSES OF CORN AS AFFECTED BY N-(3,4-DICHLOROPHENYL) METHACRYLAMIDE

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ABSTRACT

Some effects of N-(3,4-dichlorophenyl) methacrylamide (dicryl) on corn were investigated. Dicryl caused a slight reduction in length of corn coleoptiles while IAA caused an appreciable increase in elongation. The herbicide had no influence on elongatiom of coleoptiles which were also treated with IAA. It was found that both roots and shoots were affected when dicryl was applied to roots of corn.

Plant material for the remainder of the investigation was obtained by treating 5 to 6 day old corn seedlings which were grown in soil in metal flats with 0 and 3 lb/A dicryl. It was found that dicryl caused a sudden decrease in the respiration of corn leaf tissue. The rate of respiration of control tissue steadily decreased during the testing period. Low rates of DNP caused stimulation of respiration while higher rates caused inhibition. Treated tissue was much more sensitive to DNP than control tissue.

Phenol oxidase was the most active of the terminal oxidases. Negative results were obtained for the participation of the cytochrome system in the respiration of corn. Homogenates from corn tissue readily oxidized ascorbate but the oxidation was thought to be of a nonenzymatic nature.

Dicryl caused little effect on the activity of catalase 2 days after treatment, but 6 days after treatment it caused a 50 per cent reduction in activity. The herbicide had essentially the same effect on peroxidase and glycolic acid oxidase activity.

Three to 4 days after corn was treated with dicryl there was a sudden increase in the oxidation of ascorbic acid which continued to

rise throughout the testing period. The oxidation of ascorbate by homogenates from both control and treated tissue was slightly increased by boiling as well as by the addition of $CuSO_4$. Heavy metal inhibitors affected the oxidation of ascorbate very little; however, iodoacetate caused considerable inhibition. The batch treatment of supernatants with a cation exchange resin had little or no effect on the oxidation of ascorbate and the possibility of ascorbate serving as a source of electrons for the reduction of quinones proved negative. It was also determined that neither dehydroascorbate nor hydroquinone could substitute for ascorbate.

Dialysis studies indicated considerably more nitrogen was lost from dialyzed treated than from dialyzed control supernatants. When homogenates were fractionated by centrifugation, it was found that considerably more nitrogen was precipitated in control than in treated homogenates. In the same experiment it was found that centrifugation had little effect on the oxidation of ascorbate.

Some of the techniques utilized in the investigation were checked on cucumber ascorbic acid oxidase. The oxidation of ascorbate by cucumber extract could be inhibited by boiling and by 0.2 mM dieca.

The oxidation of ascorbate was affected very little when alanine, asparagine, and glutamine were added to homogenates at low concentrations but at high concentrations (0.1M) glutamine and asparagine caused some inhibition. The effect of glutathione on the oxidation of ascorbate was the same as that encountered with the higher concentrations of amino acids.

Supernatants (1,000 xg) from homogenates were fractionated by the use of a column of celite. Some fractions, which were of a brownish color, oxidized ascorbate quite rapidly. The color was separated from

ascorbate activity by chromatography on a 98 per cent celite - 2 per cent charcoal column. Copper greatly increased the oxidation of ascorbate in all fractions from the celite - charcoal column except the ones which exhibited ascorbate activity prior to the addition of copper and the ones which contained the brown color.

INTRODUCTION

Due to the ever increasing cost of labor more efficient farming methods and techniques have been developed. As a result the last decade has seen the science of chemical weed control grow tremendously. Numerous herbicides are now on the market for various types of weed control. These herbicides have been developed so rapidly that there has not been a sufficient amount of basic research done on many of them. The gross morphological effects of most of these herbicides, however, have been studied and measured. It is known rather accurately how most herbicides will affect the external characters of plants, but the knowledge about how any given herbicide will affect a given plant the way it does is very limited.

The purpose of this investigation was to obtain some information on the effects of N-(3,4-dichlorophenyl) methacrylamide (dicryl) on the growth and some of the common enzyme syatems of corn. This study was designed to follow very closely the work by Bingham (1960) who measured some of the effects of dicryl on the cotton plant.

Since cotton is relatively tolerant to the herbicide and corn is rather sensitive, it was hoped by comparing the results of these studies that some insight might be gained as to the nature of the mechanism of action of this herbicide. In addition to the information obtained on the mechanism of action of dicryl, it is hoped that some of the techniques utilized in this study will also be useful for future studies of a similar nature.

LITERATURE REVIEW

Effects of Dicryl on Plants

Porter et al. (1960) reported promising results for the use of N-(3,4-dichlorophenyl) methacrylamide (dicryl) as a potential post-emergence herbicide for use in cotton. These workers found when sprays of dicryl were semi-directed under the cotyledons of young cotton, maximum weed control would be obtained with little injury to the cotton. It was also determined that the most effective rate of dicryl was 3 to 4 1/2 lbs/A applied in two applications.

Moreland and Hill (1960) reported that dicryl caused a 50 per cent inhibition of the Hill reaction in isolated turnip-green chloroplasts at concentrations below 10^{-6} M.

Bingham (1960) and Bingham and Porter (1960a) have reported some effects of dicryl on early growth and development of cotton. These workers found that the development of cotyledons and true leaves was markedly suppressed by dicryl treatment. The maximum suppression of cotyledon weight was obtained when treated at the onset of most rapid growth. Bingham (1960) also reported that due to dicryl treatment the nitrogen content in cotyledons did not decrease as it did in the control.

Dicryl was found to suppress the elongation caused by IAA in cotton hypocotyls (Bingham, 1960). Bingham also studied the effects of dicryl and IAA on the roots of cotton. He found that both of these compounds caused a reduction in elongation of roots. From these results, he concluded that dicryl could possibly act as a weak auxin.

Bingham (1960) and Bingham and Porter (1960b) have also reported some effects of dicryl on certain respiratory enzymes of cotton. These workers found that respiration of cotton cotyledons declined during their developing period and dicryl caused only a slight decrease in respiration of these cotyledons. Bingham found that ascorbic acid oxidase was very active in the cotton plant while there was little or no cytochrome oxidase activity. Phenol oxidase activity was intermediate between ascorbic acid and cytochrome oxidase. The most significant effect of dicryl found on these oxidases was a reduction in ascorbic acid oxidase activity.

Funderburk and Porter (1960) investigated the oxidation of ascorbic acid by control and dicryl treated corn tissue. They found that ascorbic acid was oxidized at the same rate by both tissues until 3 to 4 days after treatment at which time the treated tissue started oxidizing ascorbic acid at a much higher rate.

A possible terminal oxidation system containing glycolic acid oxidase was also investigated by Bingham (1960). He presented evidence that this enzyme was very active in cotton tissue and dicryl had no apparent effect on its activity. He also found that catalase and peroxidase activity remained constant following dicryl treatment while it increased in control tissue.

Ascorbic Acid Oxidase and Ascorbic Acid

Szent-Gyorgyi (1930) reported that cabbage leaves contained an enzyme "hexoxidase" which catalyzed the direct aerobic oxidation of "hexuronic" acid. When "hexuronic" acid was identified as L-ascorbic acid, the enzyme became known as ascorbic acid oxidase. Dawson (1950) found that electrophoretic and ultracentrifuge data were in good

agreement with the view that ascorbic acid oxidase is a specific copper protein having a molecular weight of about 150,000 and containing six copper atoms per molecule. According to Mapson (1958), this enzyme is usually considered to be soluble. Waygood (1950), Newcomb (1951), and Bingham (1960), however, have shown that ascorbic acid oxidase is associated with the "cell wall" fraction in wheat, tobacco pith cells, and cotton. Mapson (1958) reported that unlike other copper proteins, ascorbic acid oxidase is very substrate specific.

Ascorbic acid, the substrate for ascorbic acid oxidase, occurs as such and in its oxidized form dehydroascorbic acid in nearly all plant tissues, but its concentration within different tissues of the same plant varies over a wide range (Mapson, 1958). Mapson reported that within the single plant ascorbic acid is mainly concentrated in regions of high metabolic activity.

Ascorbic acid is not autoxidizable within the physiological range of pH, so catalysts are essential for any reaction between it and oxygen (Mapson, 1958). Ascorbic acid oxidase, the main catalyst for this oxidation, has been mentioned above. Mapson (1958) reported that polyphenol-oxidase, laccase, cytochrome oxidase, and peroxidase will also catalyze the oxidation of ascorbic acid.

There are two reports of atypical ascorbic acid oxidases. One was found to occur in Myrothecium verrucaria by Mandels (1953) and a similar one in Physarum polycephalum by Ward (1955). Mandels used spores of Myrothecium verrucaria as a source of ascorbic acid oxidase. He reported that the enzyme was located at the surface of the spores since it was inactivated after a 30 second exposure to 0.1 N HCl. He found that exposures to the acid for as long as 40 minutes had only very little effect on respiration. Mandels reported that the enzyme

was resistant to cyanide, azide, sulfide, dieca, phenylthiourea, and 8-hydroxyquinoline; however, it was easily inactivated by boiling. This enzyme was also specific for L-ascorbic acid and would not oxidize D-arabo-ascorbic acid as will the ascorbic acid oxidase from cucumber (Snow and Zilva, 1938).

Ward (1955) used the mycelia of Physarum polycephalum as a source of his atypical ascorbic acid oxidase. This enzyme appeared to be similar to the one Mandels (1953) reported on with the exception that it would oxidize both L and D forms of ascorbic acid.

There are references in the literature to the inhibition of ascorbic acid oxidase by naturally occurring substances. Damordaran and Nair (1936) isolated a tannin from the Indian gooseberry, Phyllanthus emblica, which inhibited the oxidation of ascorbic acid in the press juice. Since the protective effect of this substance could be "overridden" by the addition of Cu, they concluded that its action depended on the suppression of metal catalysis. On the other hand, Giri and Krishnamurthy (1940) separated a substance from the juices of Cucumis sativus, Cucurbita maxima, and Luffa acutangula which prevented the oxidation of ascorbic acid even in the presence of Cu.

Hooper and Ayres (1950) have found that black currants, a fruit in which ascorbic acid is remarkably stable, contained a substance which inhibited the oxidation of ascorbic acid by the polyphenolase system of apples. The protective action was found to be associated with a red pigment and a yellow pigment. Jackson and Wood (1959) have reported recently on the presence of substances in rose hips, Rosa canina, which inhibited the oxidation of ascorbic acid. These workers found there was a marked reduction in the oxidation of ascorbic acid caused by the rose hip extract in the following oxidizing systems;

cauliflower ascorbic acid oxidase, apple polyphenolase, horseradish peroxidase, and $CuSO_4$. Jackson and Wood (1959) reported there were two substances which caused inhibition, and in paper chromatographic studies one of these remained at the starting point while the other one had an R_f value of 0.5.

Inactivation of ascorbic acid oxidase during the progress of the oxidation of ascorbic acid has been noted by Joselow and Dawson (1951b). This inactivation has been explained as resulting from two causes: (a) inactivation due to enviornmental conditions during the reaction, protection against which was afforded by inert proteins such as egg albumen and gelatin, and (b) inactivation due to some factor inherent in ascorbic acid-ascorbic acid oxidase reaction sensitive only to haem containing proteins, such as catalase or peroxidase. Frieden (1953) showed that the activating and protective influence of thyroxine on the enzyme reported by Gemmill (1951) was not specific but was shown by any effective cupric ion complexers such as ethylenediamine tetra-acetic acid, cyanide, diethyldithiocarbamate, cysteine, and other amino acids. Mapson (1958) reported that chemical compounds which activate or protect the enzyme, or both, can be divided into two groups: (a) those which activate or protect at all concentrations, and (b) those which protect at low concentrations but inhibit at higher concentrations. The first group include ethylenediamine tetraacetic acid, cysteine, nucleic acid, nucleotides, and proteins and the second is comprised of cyanide, diethyldithiocarbamate and 8-hydroxyquinoline. These latter stimulate the activity at levels of 10^{-6} to 10^{-7} M but inhibit at concentrations of 10^{-4} M or higher. Frieden (1957) observed that the enzyme was sensitive to organic mercurials and the resulting inhibition could be reversed by either cysteine or reduced

glutathione, which led him to suggest that it was a sulfhydryl dependent enzyme.

Joselow and Dawson (1951a) found that there was an exchange of radioactive Cu ion with that of the enzyme during the course of the reaction. This finding confirmed the fact that inactivation of the enzyme was not due to a loss of Cu.

Mapson (1958) reported that ascorbic acid and dehydroascorbic acid, the oxidized form of ascorbic acid, are interconvertible in plant tissues. He is of the opinion that the concentration of dehydroascorbic acid in fresh tissue is of the order of 5 per cent or less of the total ascorbic acid content. He further stated that this balance in the plant cell may be disturbed by a number of factors such as: (a) mechanical damage, (b) action of substances causing cellular disorganization, or (c) by the action of specific enzymic poisons.

At the present time there is much controversy over the possibility that ascorbic acid oxidase may act as a terminal oxidase. Of the three oxidases, ascorbic acid oxidase, cytochrome oxidase, and polyphenoloxidase, James and his colleagues (1953, 1955) were able to demonstrate only the first in 10 to 17 day-old barley roots. During the development of these roots there appeared to be a gradual replacement of cytochrome oxidase by ascorbic acid oxidase, as evidenced by the fall in sensitivity of the respiration to CO and a rise in sensitivity towards sodium diethyldithiocarbamate which pointed to a progressive change from an iron catalyzed to a copper catalyzed system. These conclusions can be questioned on the basis of conflicting reports on the sensitivity of ascorbic acid oxidase to CO (Mapson, 1958).

Through the use of several inhibitors, Farkas and Kiraly (1955) and Kiraly and Farkas (1957) reached the conclusion that cytochrome

oxidase was the main terminal oxidase in healthy wheat leaves, but in leaves infected with stem rust, the enhanced respiratory activity became highly sensitive to copper chelating agents and was paralleled by an increase in ascorbic acid oxidase activity. These authors suggested that ascorbic acid oxidase may be present in healthy plants in an inactive state but only becomes operative in infected plants and is the terminal oxidase of the parasitically stimulated respiration.

Thimann et al. (1954) have offered evidence that ascorbic acid oxidase is not of major importance as a terminal oxidase on the basis of the low affinity of the enzyme for oxygen. Bonner (1957) concluded from this information that the general thesis of the participation of ascorbic acid oxidase in catalyzing the transport of electrons to molecular oxygen in plant tissue must be abandoned. Mapson (1958) is of the opinion that this would be true only if the oxidase catalyzed the rate limiting step at all oxygen tensions, and this seems unlikely in view of our knowledge that the adenosine triphosphate (ATP) turnover probably governs this phenomenon.

Mapson (1958) concluded that at the present time it is still uncertain whether ascorbic acid oxidase ever functions as a terminal oxidase. He further stated that most observers would agree that the major terminal oxidase in plants generally is cytochrome oxidase, but the evidence does not exclude the possibility that in some tissues at different stages of development or under certain enviornmental or pathological conditions part of the respiration may be mediated via ascorbic acid oxidase.

Mapson (1958) has reviewed the possibility of ascorbic acid functioning in respiration in an electron transferring system. Such a system involving triphosphopyridine nucleotide (TPN) and reduced

glutathione was demonstrated in pea seed extracts by Mapson and Goddard (1951). Extracts from this tissue contained both malate and isocitrate dehydrogenase enzymes which reduce TPN, together with glutathione and dehydroascorbic acid thus forming oxidized glutathione and ascorbic acid. Mapson and Moustafa (1956) have extended this work with pea seedlings and shown that hydrogen was transferred from substrates of TPN linked dehydrogenases to molecular oxygen. The enzymes concerned in this pathway included dehydrogenase enzymes, glutathione reductase, dehydroascorbic acid reductase, and ascorbic acid oxidase. From the evidence available at the present time, Mapson (1958) concluded that if ascorbic acid acts as a respiratory carrier in vivo it is positioned between either DPN or TPN and the terminal oxidase.

The effect of ascorbic acid as a hydroxylating agent in scorbutic animals has been known for some time (Sealock and Silberstein, 1939). When tyrosine was administered to scorbutic guinea pigs, p-hydroxyphenyl pyruvic and p-hydroxyphenyl lactic acids were recovered from the urine, but these compounds were not seen if ascorbic acid was given simultaneously with the tyrosine.

Udenfriend et al. (1954) and Brodie et al. (1954) found that a model system consisting of ascorbic acid, iron, and oxygen could catalyze the hydroxylation of many aromatic compounds. This reaction was not specific for L-ascorbic acid since dehydroascorbic acid, diketogulonic acid, dihydroxymaleic acid, alloxan, and several other compounds could replace the vitamin. The exact nature of the hydroxylating agent is not known. Udenfriend et al. (1954) obtained evidence that it was not due to any of the constituents of the model system. These workers concluded that hydroxylation appeared to be mediated through a reaction product of hydrogen peroxide with ascorbic acid. Mapson (1958) reported

that at present there is no evidence of the existence of any hydroxylating enzyme in plants which requires ascorbic acid for its activity; however, the possibility of such a system as described above participating in melanin formation and in the production of many phenolic derivatives in plants is obvious.

Phenol Oxidase

Bonner (1957) used the name "phenol oxidase" to include all terminology which described the enzyme that catalyzes the oxidation of mono- and ortho- diphenolic substances. Therefore, tyrosinase, polyphenol oxidase, "DOPA oxidase", potato oxidase, catechol oxidase, etc., are covered in the generic term phenol oxidase.

The first report of phenol oxidase was made by Boswell and Whiting (1938). These workers added catechol to respiring potato slices and found that there was marked increase in oxygen uptake. They concluded that phenol oxidase was involved in approximately two-thirds of the total respiration of potato tuber slices.

Bonner (1957) reported that in spite of continued interest in the enzyme throughout a great many years, the present-day knowledge concerning the properties of phenol oxidase is distressingly small. The enzyme has been reported from various sources (Bonner, 1957); however, it varies somewhat as to color and to copper content. Bonner (1957) is of the opinion that regardless of these variations there can be no question that it is the same enzyme in all cases. Mallete et al. (1948) found that the highly purified enzyme had a molecular weight of 100,000, a figure which corresponds to a copper content of four atoms of copper per mole.

Bonner (1957) reviewed the literature on whether phenol oxidase

catalyzed only the oxidation of monophenols or ortho-diphenols or both. He concluded that the majority of the information favored the latter possibility.

It has been known for some time that this enzyme was in part responsible for the darkening of plant tissues. Lerner and Fitzpatrick (1950) worked out the pathway of melanin formation and demonstrated exactly where phenol oxidase functioned in the system.

Cytochromes

The presence of cytochromes in higher plants has been known for some time (Keilin, 1925). Hill and Hartree (1953), Hartree (1953), and Smith and Chance (1958) have all published extensive and critical review articles on cytochromes in plants. The cytochrome system is considered by some to be the only terminal oxidase system which operates in plant tissue; however, Smith and Chance (1958) reported that it is not always clear what is meant by the term "terminal oxidase". They stated that possibly one oxidative pathway may be the one which functions in energy-yielding reactions, while other oxidases have synthetic or other functions.

Smith and Chance (1958) reported that there is abundant evidence for cytochrome oxidase in homogenates of many plant tissues and it has also been reported to be lacking for some. They point out that the assay for cytochrome oxidase is fraught with difficulties. Hill and Hartree (1953) have pointed out some of the difficulties in using manometric techniques for cytochrome oxidase assay in plant homogenates.

The cyanide resistant respiration found in the spadix of Arum is a typical example of the complexity of the cytochrome system. James and Beevers (1950) reported that the exceptionally high rate of

respiration in Arum spadix was insensitive to cyanide whereas respiration in all other parts of the plant was low and could be markedly inhibited by cyanide. James and Beevers (1950) concluded from this information that a flavoprotein could be the major terminal oxidase.

Hackett and Simon (1954) found that particles of the Aroid spadix could oxidize succinate and alpha-ketoglutarate. At this point they began to suspect that flavoproteins were not the terminal oxidase as had been proposed. Bendall and Hill (1956) were able to determine the presence of cytochromes a, b, and c in the Aroid spadix. The cytochrome b these workers found was different from the normal cytochrome b and they labeled it cytochrome b_7. Bendall and Hill (1956) determined that this cytochrome b_7 was present only in the sterile portion of the flower and the remainder of the plant contained the normal cytochrome b. Hackett and Haas (1958), and Chance and Hackett (1959) have reported the finding of cytochrome b_7 in Aroid spadix. At the present time these workers are all in agreement that the cyanide resistant respiration in the Arum spadix is mediated through cytochrome b_7.

Catalase and Peroxidase

Catalase is a metalloprotein enzyme with hematin as its prosthetic group. This enzyme catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen (Lardy, 1950). It is believed that this enzyme is of wide spread occurence. Bonner (1950) reported that catalase is found in all higher plants and Clayton (1959) has recently extracted, purified, and characterized catalase from the bacterium, Rhodopseudomonas spheroides.

Catalase was crystallized for the first time by Sumner and Dounce (1937) from beef liver; it was the first enzyme containing iron to be

isolated in a crystalline form. Sumner and Dounce found that the enzyme contained 0.1 per cent iron and its isoelectric point was found to be at pH 5.7.

In regards to the effect of temperature on catalase, Sizer (1944) reported that below 53°C the rate of the enzyme catalyzed reaction increased with temperature. Above 53°C the enzyme catalyzed reaction dropped off due to heat inactivation of the enzyme. Appleman and Pyfrom (1955) studied the effect of light on the catalase activity of several species of grains. They found that catalase activity was greatly depressed by blue light and elevated by red light.

The enzyme peroxidase, in the presence of hydrogen peroxide, catalyzes the oxidation of many phenols and aromatic amines (Lardy, 1950). Bonner (1950) reported that peroxidase occurred universally or nearly so in plant tissues.

Palmer and Porter (1959) indicated that amitrol treatment of nut grass tubers inhibited germination and apparently held the activity of peroxidase at about the same level of activity as the dormant tubers. The peroxidase level increased in germinated tubers to 2.5 times that in dormant tubers.

Glycolic Acid Oxidase

This enzyme was first discovered by Clagett et al. (1949). It was shown to catalyze the oxidation of glycolic and L-lactic acids and to be of wide spread occurrence in the green parts of many plants. Tolbert et al. (1949) reported the optimum pH for the enzyme appeared to be 7.8 to 8.6 and it did not break down on dialysis. At this time the enzyme was referred to as alpha hydroxy acid oxidase.

Zelitch et al. (1953, 1957, 1958, 1960) have done a considerable

amount of work with this enzyme which is now referred to as glycolic acid oxidase. Zelitch and Ochoa (1953) isolated glycolic acid oxidase from spinach leaves in a highly purified form. The prosthetic group of the enzyme was shown to be riboflavin phosphate.

Glyoxylic acid reductase was also isolated from spinach (Zelitch, 1953). This enzyme catalyzes the reduction of glyoxylic to glycolic acid by reduced pyridine nucleotides. In the presence of catalytic amounts of DPN or TPN and glyoxylic or glycolic acid, glyoxylic acid reductase can act, together with glycolic acid oxidase, as a hydrogen carrier system from reduced pyridine nucleotide to molecular oxygen. Zelitch (1953) suggested that this system may function in the respiration of green leaves. In order to evaluate this assumption it was necessary to find an inhibitor of glycolic acid oxidase. Zelitch (1957) found that the alpha hydroxysulfonates were very effective and highly specific competitive inhibitors of this enzyme. He was able to show, through the use of these inhibitors, that the glycolic acid content of excised leaves of several plants would increase to more than ten times the normal level. This phenomenon took place only in sunlight. Zelitch measured the accumulation of glycolic acid in spinach, tomato, bean, and corn plants. His results showed less accumulation of glycolic acid in corn than in any of the other three plants.

Zelitch and Barber (1960) have recently checked the possibility of oxidative phosphorylation with glycolic acid as a substrate. He concluded that spinach particles which were capable of carrying out oxidative phosphorylation with other substrates could not do this with glycolic acid as the substrate.

METHODS AND MATERIALS

I The Herbicide

Dicryl is a white powder which is less than 10 ppm soluble in water. It is soluble in isophorone, pyridine, acetone, and various alcohols.

The commercial emulsifiable concentrate of dicryl contains isophorone, wetting agent, and an emulsifier. The commercial preparation was used to treat the plants for these studies unless stated otherwise.

II Growth of Plant Material

Dixie 18 corn seeds were planted in metal flats which contained a sandy loam soil. The flats were maintained in the greenhouse for 5 to 6 days, at which time they were sprayed with 0 and 3 lb/A dicryl in 40 gallons of water. Immediately after the spraying operation the plants were placed in a growth room where the temperature was maintained at 30°C. Light in the growth room consisted of a mixture of fluorescent tubes and incandescent bulbs and the intensity at soil level was 500 foot candles.

III Preparation of Whole Tissue for Respiratory Experiments

A. General Procedure

Leaves were cut from the corn plant, and if no subsequent treatment was to be applied, the midribs were torn from the leaves and the leaf sections cut into segments approximately one-fourth inch in length. These leaf pieces were weighed and transferred to Warburg vessels.

In certain experiments involving the use of respiratory inhibitors,

the inhibitor was contained in the sucrose-phosphate buffer which was added to each flask.

B. Vacuum Infiltration

After leaves had been removed from the plant as described above they were weighed and placed in a beaker which contained the desired solution to be vacuum infiltrated. These beakers were placed in a vacuum desiccator attached to a water aspirator. A vacuum was applied to the desiccator and then slowly released. The vacuum was then re-applied for 10 minutes, slowly released, the tissue removed from the solution, blotted, and placed in Warburg flasks with appropriate solutions.

C. Treatment with Carbon Monoxide

Whenever carbon monoxide was used as an inhibitor, it was used as a mixture which contained 95 per cent carbon monoxide and 5 per cent oxygen. The treatment with carbon monoxide was performed by flushing Warburg vessels which contained the tissue with approximately 2 liters of the gas mixture. The stopcocks were immediately closed at the end of the flushing period and the manometers placed on the Warburg apparatus.

IV Preparation of Homogenates and Supernatants

Two grams of the first and second leaves (above the coleoptile) from corn (midrib removed) were sliced into very small sections with a scalpel. These sections were placed in an omni-mixer in 10 ml of 0.05 M potassium phosphate buffer of pH 5.85. Unless otherwise stated, this buffer and pH were the same for all homogenate preparations. The mixer, which was connected to a powerstat, was operated at 90 volts for 10 minutes. The mixing vessel was surrounded by an ice bath which maintained the temperature below 5°C. At the end of the mixing period 18 additional ml of

phosphate buffer was added to the homogenate.

In certain studies the homogenate was centrifuged at 1,000 xg for 10 minutes at 0°C and the supernatant was used as an enzyme source.

V Determination of Oxygen Uptake and Carbon Dioxide Production

Respiratory activity of whole tissue and homogenates was determined using a Warburg respirometer and conventional methods described by Umbreit et al. (1957). Duplicate flasks were run for each treatment. To flasks in which oxygen uptake alone was determined, a small piece of folded filter paper and 0.15 ml of 20 per cent KOH were placed in the center well. Flasks which contained whole leaf tissue also received 2.0 ml of a buffer solution which was 0.02 M and 0.05 M with regard to sucrose and potassium phosphate respectively.

Manometer readings were generally recorded at 15 to 30 minute intervals. Oxygen uptake was usually expressed as $Q_{O_2}(N)$, which means microliters of oxygen uptake per milligram of nitrogen.

VI Estimation of Enzyme Activity

A. Phenol Oxidase

A 2.0 ml aliquot of homogenate was placed in the main compartment of a Warburg flask and 0.5 ml of 0.12 M catechol added to the side arm. Catechol was tipped into the homogenate after a 15 minute equilibration period. The catechol was prepared and adjusted to pH 5.85 shortly before it was added to the flask

B. Cytochrome Oxidase

A 2.0 ml aliquot of homogenate (prepared in buffer of pH 7.0) was placed in the main compartment of a Warburg vessel. One-half ml of 2×10^{-4} cytochrome c and 0.5 ml of 0.12 M p-phenylenediamine were placed in separate side arms of the vessel and, following equilibration, tipped

into the homogenate. Immediately before addition to the flask, the p-phenylenediamine solution was adjusted to pH 7.0.

C. Glycolic Acid Oxidase

For these studies the supernatant from a homogenate centrifuged at 1,000 xg for 10 minutes was used as the enzyme source. A 3 ml aliquot of supernatant was added to the main compartment of the Warburg flask and 0.5 ml of 0.14 M glycolic acid or lactic acid of pH 7.8 was placed in the side arm. After equilibration, the content of the side arm was tipped into the main compartment and oxygen uptake recorded.

D. Catalase

A modified procedure of Appleman (1951) was used. The main compartment of the Warburg flask received 2 ml of H_2O_2 (final concentration 10 mM). One ml of diluted enzyme preparation (5-fold dilution) was added to the side arm. The enzyme preparation was a supernatant from a homogenate centrifuged at 1,000 xg for 10 minutes. After equilibration in a 20°C water bath, the enzyme was tipped into the main compartment and oxygen production recorded at one minute intervals for 10 minutes. The homogenate and H_2O_2 were prepared in 0.20 M potassium phosphate buffer of pH 7.0.

E. Peroxidase

The homogenate supernatant of 1,000 xg for 10 minutes was used as the enzyme source. A manometric procedure given by Ettori (1949) was used. The main compartment received the following: 0.2 ml of 5 per cent pyrogallol, 1.5 ml of H_2O and 0.2 ml of 1 per cent H_2O_2. A 0.2 ml aliquot of enzyme extract and 0.5 ml of 0.25 M potassium phosphate buffer were added to the side arm. After equilibration the enzyme was tipped into the main compartment and manometer readings recorded at one minute intervals for 10 minutes. A second set of duplicate flasks were included in

which alkali and a small piece of filter paper were placed in the center well. These additional flasks gave an estimation of the oxygen produced by catalase activity which had to be accounted for in the total gas production of the other flasks. The activity of peroxidase is expressed in terms of carbon dioxide production in the conversion of pyrogallol to purpurogallin.

VII Techniques Employed in Studying the Oxidation of Ascorbic Acid by Corn

A. Homogenates

A 2.0 ml aliquot of homogenate was added to the main compartment of a Warburg flask and 0.5 ml of 0.25 M ascorbic acid was placed in the side arm of the flask. At the end of the equilibration period the manometers were sealed and the ascorbic acid tipped into the main compartment which contained the homogenate. Experiments of this nature were conducted at a pH of 5.85 unless stated otherwise.

B. Inactivation of Enzymes by Boiling

This technique was used on several different enzymes. It consisted of heating the homogenate in a beaker over an open flame until it started to boil. Boiling was allowed to continue for 2 minutes after it was initiated. After the homogenate had cooled, it was brought back to the original volume through the addition of potassium phosphate buffer.

C. Fractionation of Homogenates by Various Speeds of Centrifugation

The International refrigerated centrifuge and the Spinco model L ultracentrifuge were employed in the fractionation of homogenates. After the homogenate was prepared it was centrifuged for 10 minutes ($0^{\circ}C$) at 1,000 xg in the International refrigerated centrifuge. The supernatant was collected from this step and placed in the Spinco model L ultracentrifuge which was run for 30 minute periods at speeds of 50,000 to 144,000 xg.

The supernatant was removed and checked for the oxidation of ascorbic acid by the technique described under "Homogenates" (IV A.).

D. Dialysis

Fifteen ml of either homogenate or supernatant was dialyzed for 18 hours in a refrigerator (10°C) against a 0.05 M potassium phosphate buffer of pH 5.85. The buffer solution was changed 4 and 8 hours after dialysis had been initiated. Dialysis was conducted in a bag of cellophane tubing. At the end of the dialyzing period the homogenate or supernatant was checked for the oxidation of ascorbic acid by the technique described under "Homogenates" (IV A.).

E. Steam Treatment of Corn Plants

In order to see what injury by means other than a herbicide would do to corn, it was decided to treat corn with steam and then check the oxidation of ascorbate 24 hours later. The treatment consisted of passing a small jet of steam slowly over each leaf of 6 day old corn plants. The plants were then placed in a growth chamber until time to check for ascorbate activity.

F. Cation Exchange Resin

Supernatants (1,000 xg) from treated and untreated corn tissues were batch treated with Amberlite IR 120 in order to remove any cations that might affect the oxidation of ascorbic acid. The cation exchange resin was prepared according to the method described by Joselow and Dawson (1951b). The resin was removed from the mixture by centrifugation (500 xg). The supernatant was then checked for ascorbate activity.

G. Ashing of Homogenates

A 10 ml aliquot of homogenate was placed in a crucible. The crucible was heated slowly until the liquid contents had evaporated. Maximum heat was then applied to the crucible for 2 hours with an ordinary Bunsen

burner. The flame was then removed, the crucibles were allowed to cool and were brought back to the original volume with potassium phosphate buffer. The ashed homogenate was then checked for ascorbate activity by the usual method.

H. The Use of Inhibitors

Potassium cyanide, sodium diethyldithiocarbamate (dieca), 8-hydroxyquinoline, potassium ethylxanthate, and salicylaldoxime were all used at several different mM concentrations as possible inhibitors of the oxidation of ascorbic acid by corn tissue. All inhibitors were used at a pH of 5.85 and in addition dieca was also used at pH's of 6.5 and 7.0. According to James (1953) all of these inhibitors are good chelators of copper. These inhibitors were usually placed in the side arm and tipped into the homogenate or supernatant or in some cases they were pipetted directly into the homogenate or supernatant prior to equilibration.

Iodoacetate, a sulfhydryl inhibitor, was also used at concentrations of 0.01, 0.05, and 0.1 M.

I. Possible Substitutes for Ascorbic Acid

Since Udenfriend et al. (1954) found that there were several compounds which could substitute for ascorbic acid in a model system that catalyzed the hydroxylation of many aromatic compounds, it was decided to see if any of these would work with corn tissue. Dehydroascorbic acid and hydroquinone were tried as substitutes. They were used at the same pH and at the same concentration as ascorbic acid.

J. Extraction of Cucumber Ascorbic Acid Oxidase

In order to check on some of the techniques used in measuring the oxidation of ascorbic acid in corn tissue, it was decided to test cucumber, a well known source of ascorbic acid oxidase. Several cucumbers

were purchased from the local market, the tip was cut off one end and the cucumber was frozen with dry ice. After the cucumber had been frozen it was allowed to thaw at room temperature. The "juice" which dripped from the cucumber upon thawing was collected and saved as the source of the enzyme. The "juice" was diluted with buffer (4:1 v/v) before it was checked for ascorbic acid oxidase activity.

K. Column Chromatography

A technique for column preparation described by Neher (1959) was followed very closely. A slurry of methanol and celite (Johns-Manville) or methanol, celite, and activated charcoal (Darco G-60) was poured into a glass tube 2 cm in diameter and packed to a length of 16 cm. All operations were carried out at room temperature. Eight ml of supernatant from corn homogenates were placed on the column. The supernatant came from 2 gm of tissue which had been ground in an omni-mixer for 10 minutes at 90 V with 10 ml of 0.05 M potassium phosphate buffer of pH 5.85. The homogenate which resulted from this procedure was strained through two layers of cheese cloth and then centrifuged at 1,000 xg for 10 minutes.

In some cases the supernatant was eluted from the column with phosphate buffer and in others buffer followed by 10 per cent pyridine was used. Ten ml fractions were collected on a Reco fraction collector which was set on a volume control basis. In some cases the fractions were evaporated under vacuum at room temperature for 2 hours and in others they were placed in 400 ml beakers and allowed to evaporate to dryness overnight in an air conditioned room. This was necessary in order to remove the alcohol which caused difficulty in manometric techniques. The fractions were then brought back to the original volume with phosphate buffer solution. Each fraction was then checked for its ability to oxidize ascorbic acid.

L. Paper Chromatography

Paper chromatography was used to study the effect of dicryl on alpha amino acids. The paper used in this study was Whatman number 1. A solvent system consisting of n-butanol, acetic acid, and water (4:1:1, v/v/v) was used for one-dimensional chromatography. When two-dimensional chromatography was run, the second solvent system consisted of collidine, lutidine, and water (1:1, v/v). A 0.15 per cent ninhydrin-butanol solution was used to detect the alpha amino acids.

VIII Nitrogen Determination

Protein nitrogen in homogenates, supernatants, or extracts was determined by a modified procedure of Hiller et al. (1948).

Three ml of the nitrogen source was added to 8 ml of digestion mixture (500 ml of H_2O + 75 g of Na_2SO_4 + 500 ml concentrated H_2SO_4) in a 125 ml Kjeldahl flask. A grain of Cu SeO_4 was added to the mixture to serve as a catalyst. The sample was digested until 5 minutes after clearing with moderate heat. It was then allowed to cool and 20 ml of distilled water was added to the flask.

The sample was titrated back to the original color with dilute acid (sulfuric acid with acid factor equal to 0.124). The ml of acid required times 0.124 equaled the mg of nitrogen in the sample.

IX Straight Growth Test

The techniques used in this study were very similar to those described by Nitsch and Nitsch (1956). Dixie 18 corn seeds were soaked for 4 hours in aerated tap water. They were then placed on moist filter paper and exposed to red light in a moist chamber where the temperature was 25°C and the relative humidity was 90 per cent.

When the coleoptiles were approximately 30 mm in length, the top 3 mm

were removed and the next 10 mm served as a section for the straight growth test. The 10 mm sections were soaked in a 1 ppm manganous sulfate solution for 3 hours before the following treatments were applied: 0, 0.1, 1.0, 5.0, and 10.0 ppm indoleacetic acid (IAA) and dicryl separately and in combination. Growth measurements were made 20 hours after the sections were subjected to the various treatments. A binocular with an ocular micrometer was used for measuring length of the coleoptiles.

X The Absorption and Translocation of Dicryl When Applied to Roots of Corn

Three day old Dixie 18 corn seedlings, which were grown in soil, were transferred to Hoagland and Arnon's (1950) nutrient solution number 2. The entire experiment was conducted in a growth room. Four days after the plants were placed in the nutrient solution dicryl was applied to the solution at the following rates: 0, 1, 5, 10, 50, 100, and 500 ppm. Ten days later the plants were harvested and root and shoot dry weights taken.

EXPERIMENTAL RESULTS AND DISCUSSION

I. The Influence of Dicryl on Growth of the Corn Plant

The first studies on growth involved the effects of indole-3-acetic acid (IAA), dicryl, and combinations of both on the elongation of corn coleoptiles. The results of these tests (Figure 1) indicated that dicryl caused a slight reduction in length of corn coleoptiles. There was a definite increase in elongation of coleoptiles which were treated with IAA. This stimulation of elongation increased with increasing concentrations of IAA. It is also apparent from the data recorded in Figure 1 that dicryl had no influence on the elongation of coleoptiles which were also treated with IAA.

Bingham (1960) concluded from his work on cotton that dicryl could possibly act as a weak auxin. Since dicryl did not cause any stimulation of growth of corn coleoptiles and since it did not interfere with IAA when used in combination, it appeared not to be acting as a weak auxin in corn.

The next studies which involved the influence of dicryl on growth concerned the absorption (by roots) and translocation of dicryl. This herbicide is normally applied as a foliar spray to young weed seedlings. Its activity when applied to the above ground portions of plants has been established (Porter, 1960); however, the effect of this herbicide is not known when applied to the roots of plants.

The results of this test are illustrated in Figure 2. It is acknowledged that the last three rates applied far exceeded the solubility of the chemical in water. From the data it is apparent that both roots

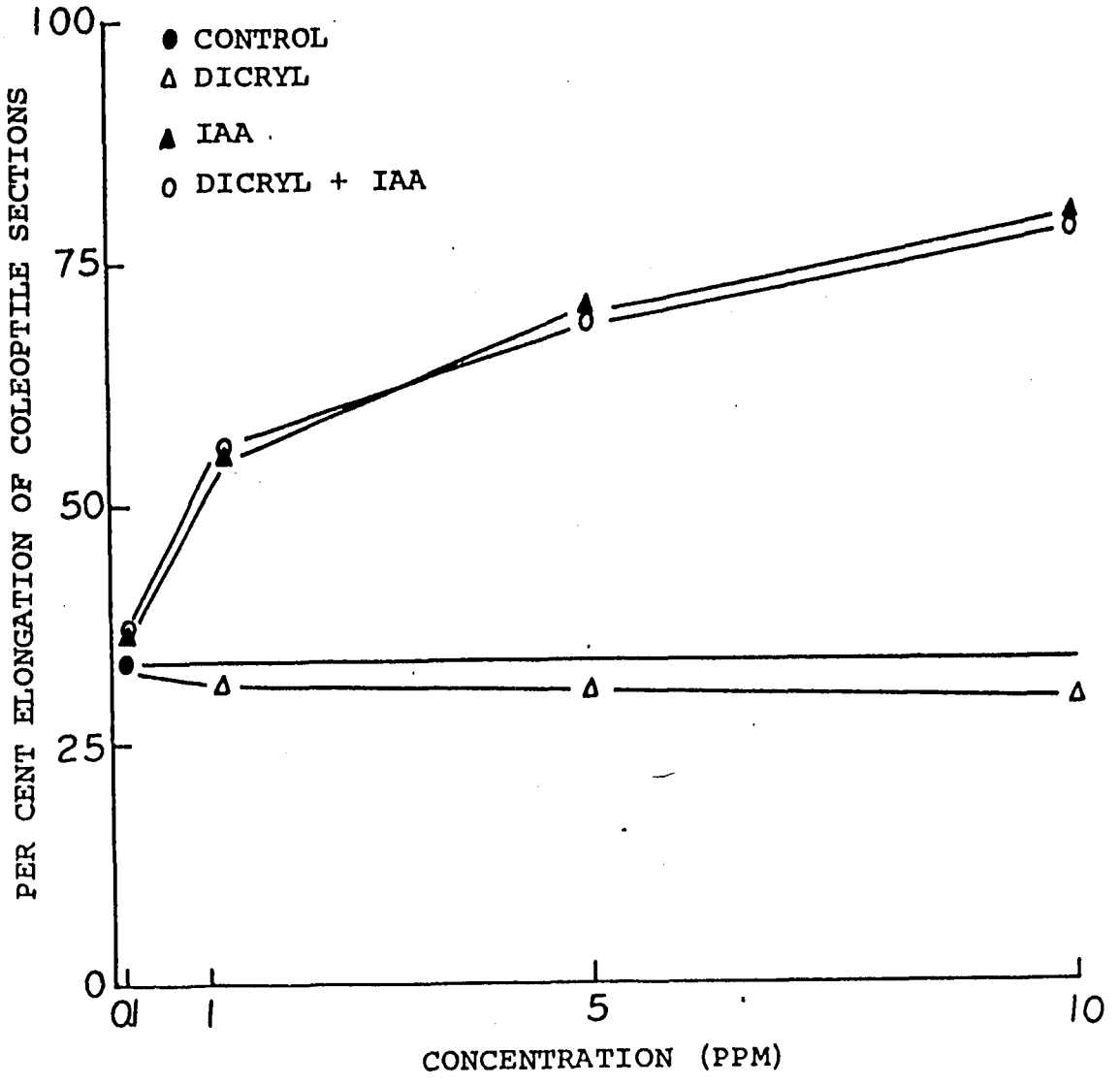


Figure 1. Some effects of dicryl, indoleacetic acid (IAA) and combinations of both on the elongation of corn coleoptiles.

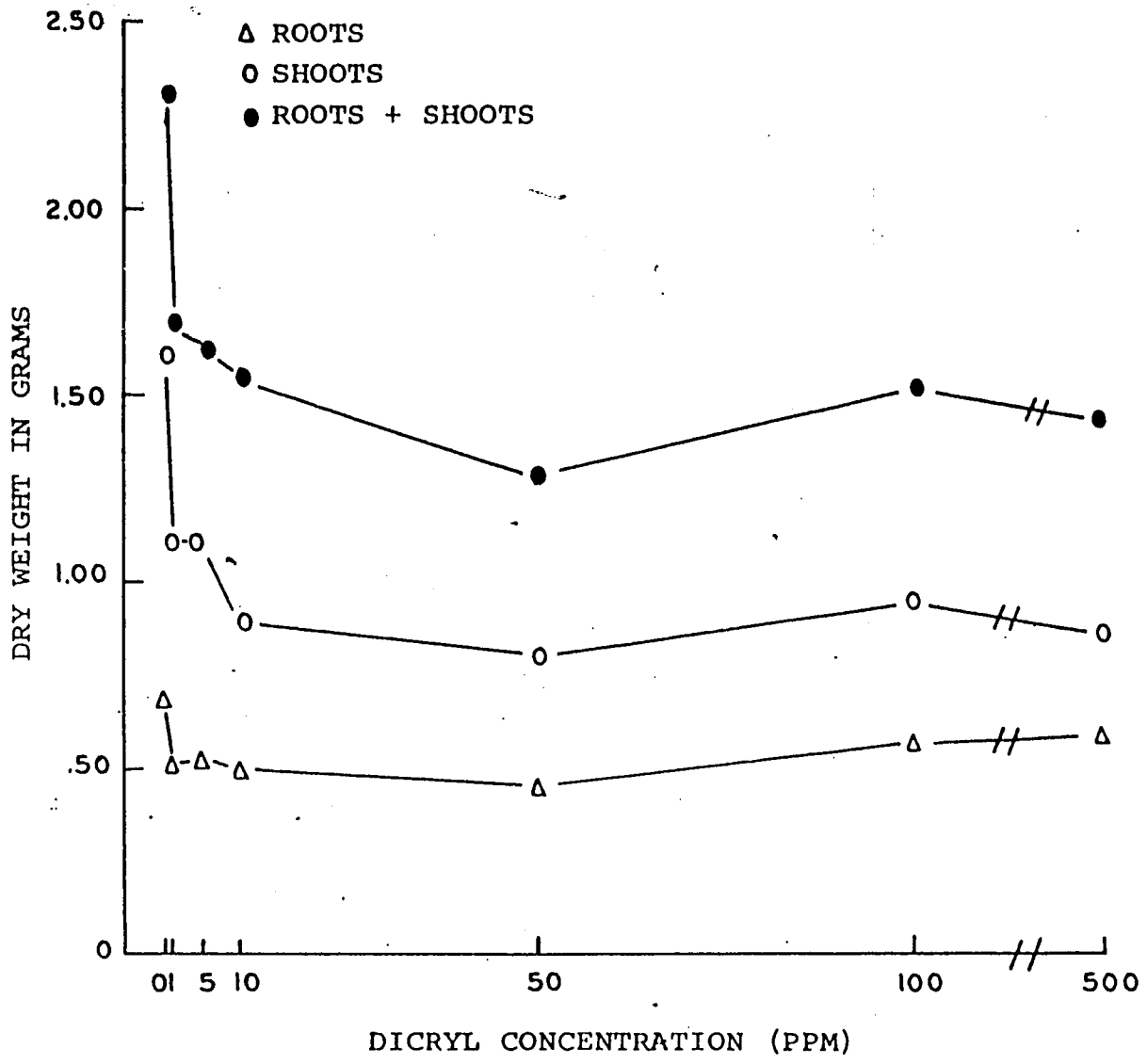


Figure 2. The influence of various concentrations of dicryl (applied to roots in nutrient culture) on the dry weight of roots and shoots of corn.

and shoots were affected by root treatment. It is also apparent that 1 ppm had a very marked effect on dry weights and the effect seemed to taper off somewhat from this point. One of the most striking things found was that 50 ppm appeared to be more toxic than either 100 or 500 ppm. This could possibly be due to the fact that at higher rates a great majority of epidermal cells are killed which could result in a decreased uptake of the chemical.

The question as to whether or not dicryl is translocated to shoots when applied to roots remains to be answered. It has been shown that shoots are affected; however, this could be a secondary effect. The herbicide could be affecting the root system only, which would cause also an effect on the shoots or it is possible that dicryl could be having a direct effect on both roots and shoots. It was noticed by the author that none of the plants in which the roots were treated with dicryl exhibited any symptoms which characterize foliar treated plants.

II. Respiratory Studies on Control and Dicryl Treated Corn Tissue

The influence of dicryl and its carrier, isophorone, on the respiration of corn was studied for an eight day period after treatment (Figure 3). It was found that the respiration of control tissue decreased steadily during this eight day period. Dicryl caused a 40 to 50 per cent reduction in respiration as soon as it was applied. The rate of respiration of dicryl treated tissue changed very little during the testing period and after eight days the respiration of treated tissue was approximately equal to that of control tissue.

Isophorone was used because it was the carrier for the commercial preparation of dicryl which was used to spray the plants utilized in all phases of this investigation. The influence of isophorone on respiration

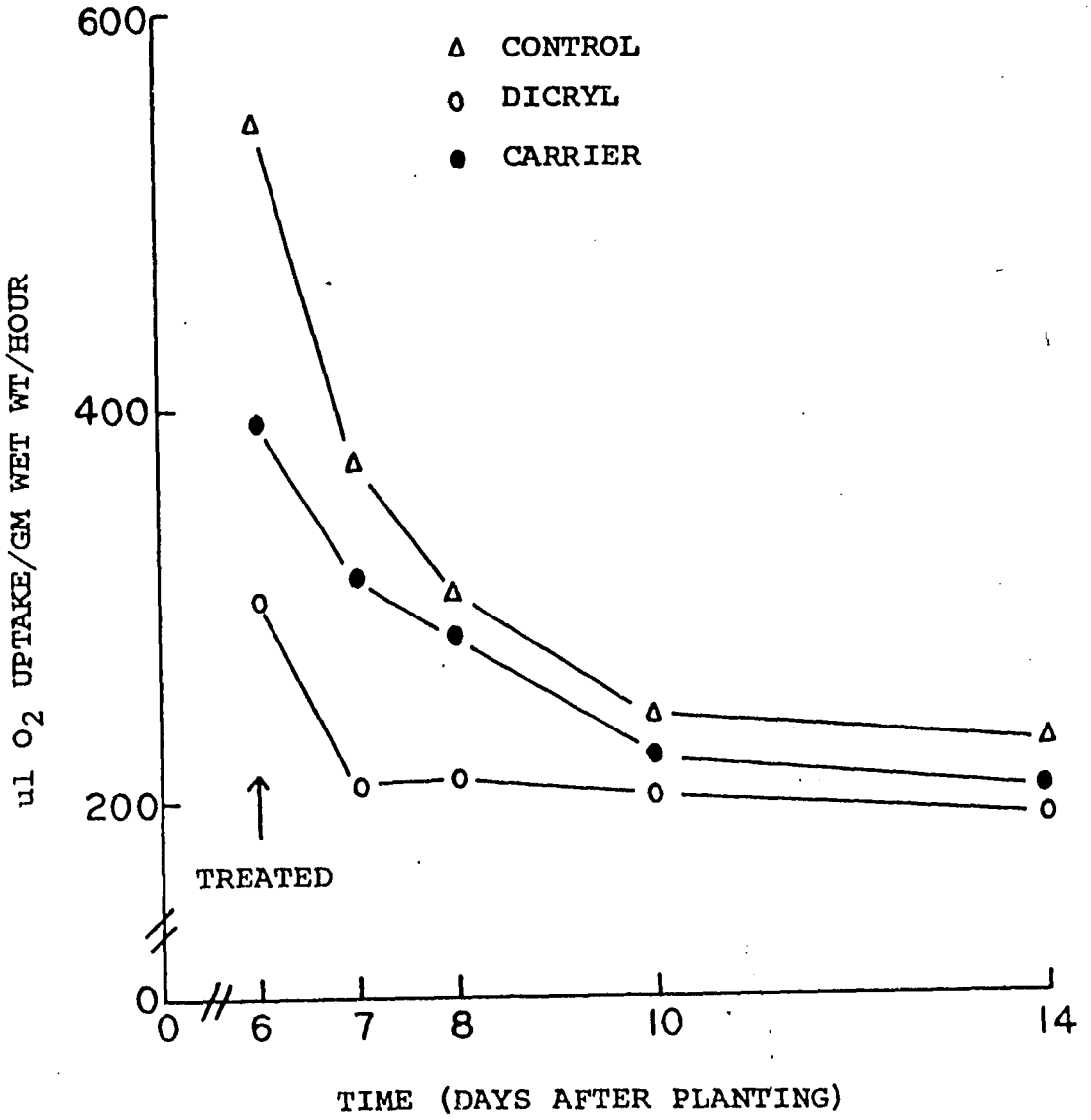


Figure 3. The effect of dicryl and isophorone (the carrier used in the commercial preparation of dicryl) on the respiration of corn leaf tissue.

was found to be intermediate between that of control and dicryl treated tissue (Figure 3).

The effect of several respiratory inhibitors on control and dicryl treated corn tissue was studied. All of these studies were made 4 to 6 days after treatment. Sodium fluoride was used at a concentration of 10 mM. According to James (1953), this compound inhibits the formation of phosphopyruvic from phosphoglyceric acid. It was found that control tissue was inhibited 35 per cent while treated tissue was inhibited 42 per cent (Figure 4A). This indicated that both tissues utilized the glycolytic pathway in their respiration and to approximately the same extent.

Iodoacetic acid was used at a concentration of 1 mM. James (1953) reported that this compound alkylated -SH groups which occur on native proteins. He stated that the most susceptible glycolytic enzyme is considered to be triosephosphate dehydrogenase. It was found that control and treated tissues were again inhibited approximately the same amount by iodoacetic acid (Figure 4B).

The third inhibitor used was sodium azide at a concentration of 1mM. The effect of sodium azide is primarily that of inhibiting metal oxidases (James, 1953). Since essentially the same amount of inhibition was obtained in both tissues, it appears that approximately the same per cent of terminal oxidation of both tissues was mediated through metal containing enzymes (Figure 4C).

The effects of 10 mM malonic acid on control and treated tissues are illustrated in Figure 4D. There was only a small amount of inhibition caused by this inhibitor. There did not appear to be any difference between control and treated tissues. The principal effect of malonic acid is in the inhibition of succinic dehydrogenase.

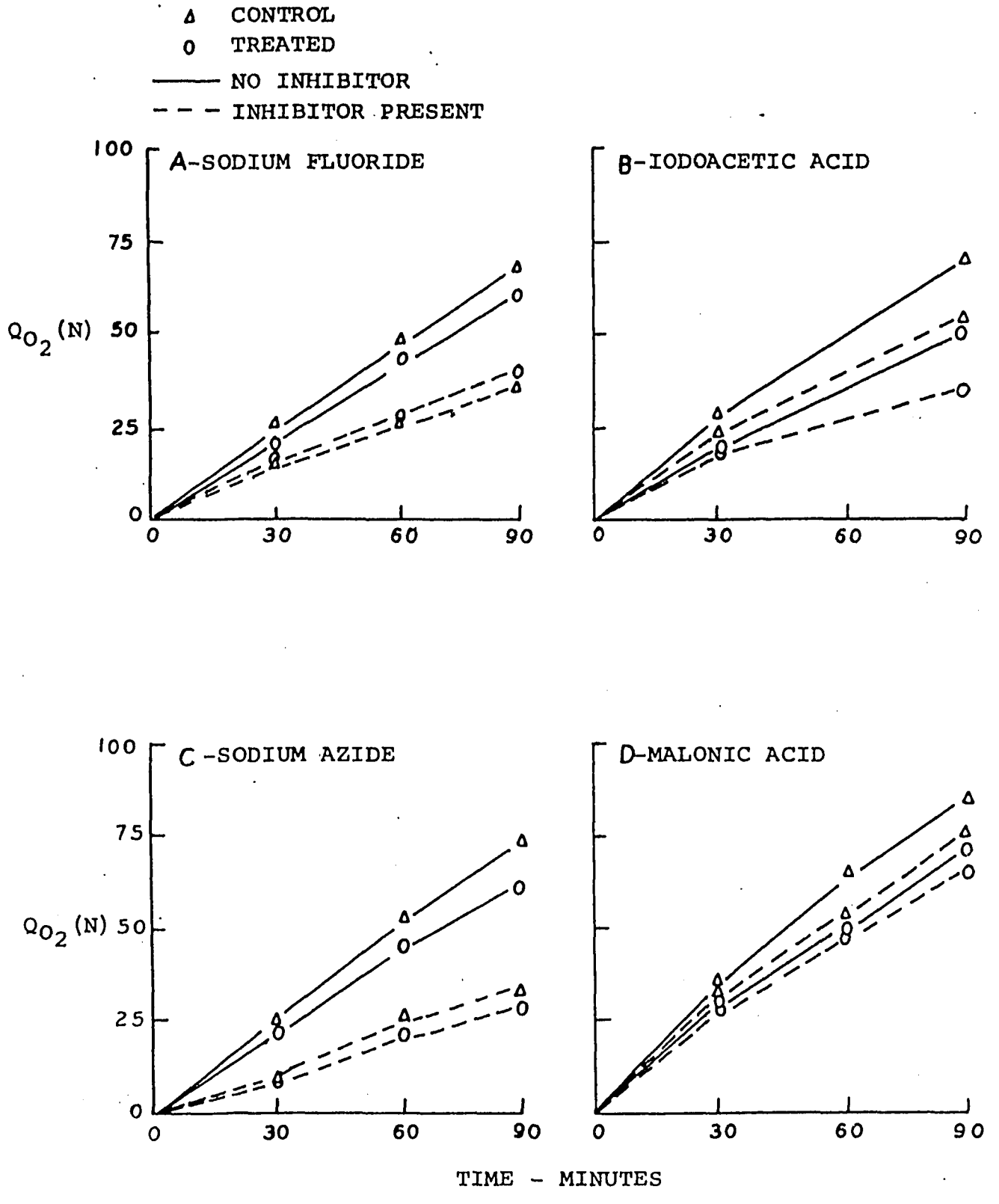


Figure 4. The effect of (A) sodium fluoride, (B) iodoacetic acid (C) sodium azide, and (D) malonic acid on the oxygen uptake of control and dicryl treated corn tissues (4 to 6 days after treatment).

The most interesting results obtained with inhibitors were recorded for 2,4-dinitrophenol (2,4-DNP). At low concentrations this material may cause stimulation of oxygen uptake while higher rates usually inhibit respiration (James, 1953). From Figure 5, it is apparent that treated tissue was much more sensitive to DNP than control tissue. This effect was measured over a series of concentrations and the relative sensitivities were found to be approximately the same with all rates. The treatment with dicryl could have caused a difference in permeability of the tissues, however, Bingham (1960) found exactly the opposite effect of DNP on cotton.

There is very little known about the mechanism of action of 2,4-DNP. The respiratory stimulation with this material is thought to be attributed to inhibition of phosphorylation and formation of energy-rich phosphates (James, 1953).

The respiratory quotient (R.Q.) for control tissue was found to be 0.89 while treated tissue was 0.77 (2 days after treatment). This indicated that more nitrogenous substances were utilized in respiration of treated tissue.

III. Studies Concerning Some of the Respiratory Enzymes in Control and Dicryl Treated Corn Tissues

The initial study which involved respiratory enzymes was designed to measure phenol oxidase and ascorbic acid oxidase activity 0 to 8 days after dicryl treatment. Catechol was used as the substrate for phenol oxidase. This enzyme was found to be quite active in corn tissue and it increased in activity until the plant was 11 days old (Figure 6). Three days later the activity of phenol oxidase had declined until it was about equal to that on the first day of measurement. Since homogenates from control and treated tissue oxidized catechol at about the same rate it

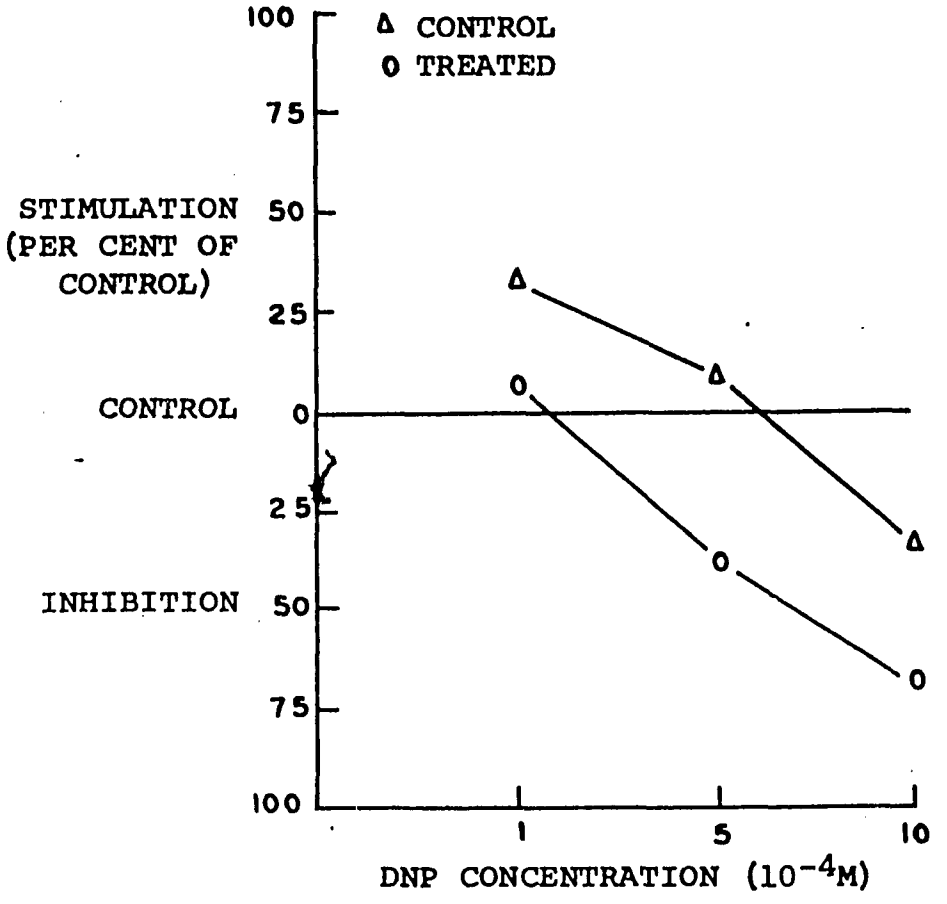


Figure 5. Some effects of various concentrations of DNP on the respiration of control and dicryl treated corn leaf tissue.

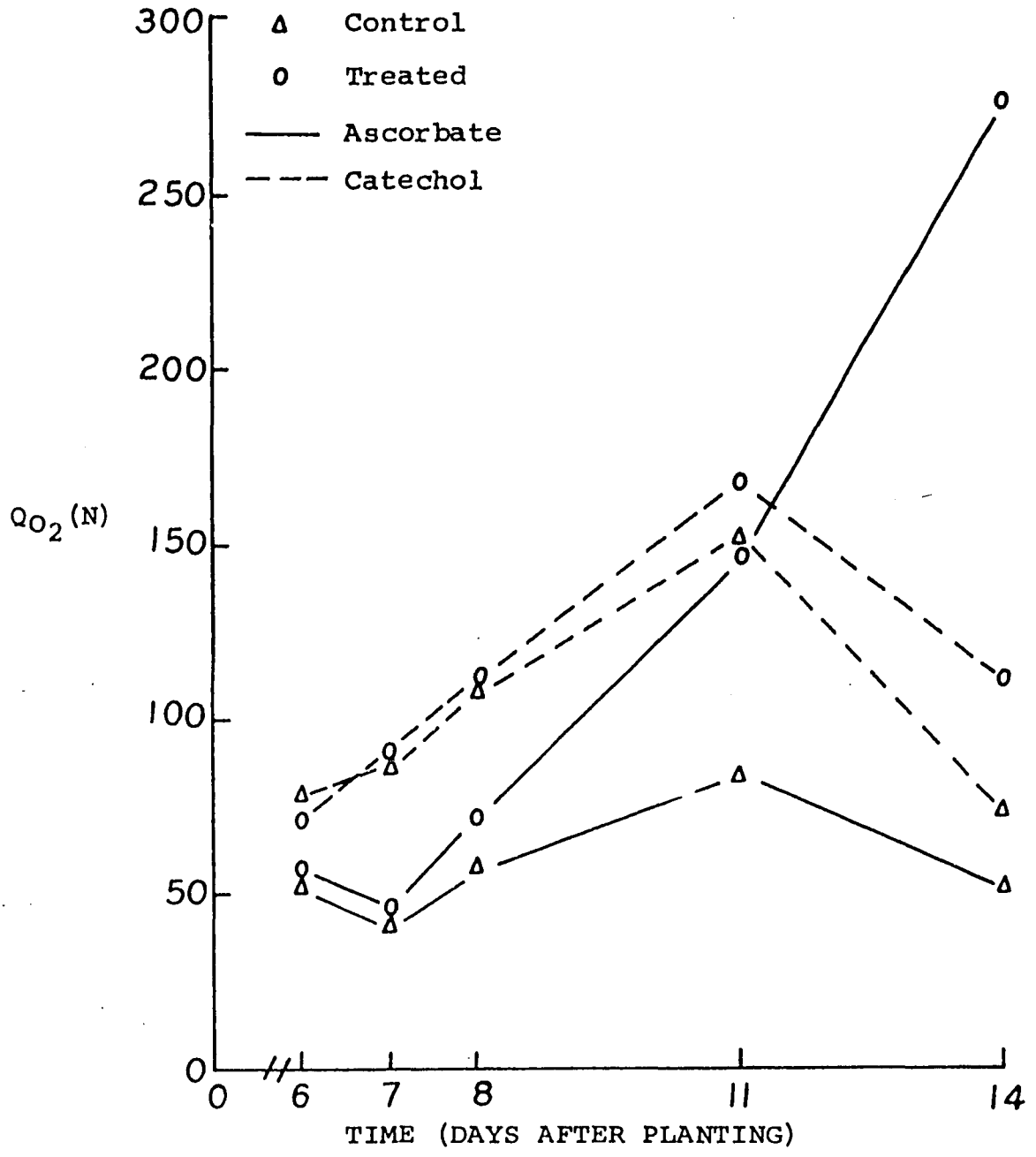


Figure 6. The oxidation of catechol and ascorbic acid by corn tissue which had been treated with 0 and 3 lb/A dicryl.

was concluded that dicryl had very little or no effect on the activity of this enzyme. McWhorter and Porter (1960) have also reported that phenol oxidase was quite active in young corn plants.

Additional evidence of the enzymatic nature of the substance which caused the oxidation of catechol is offered in Figure 7. After the homogenate had been boiled for 2 minutes it completely lost its ability to oxidize catechol.

Ascorbic acid was used as a substrate for ascorbic acid oxidase. There appeared to be no difference in the oxidation of ascorbic acid by control and treated tissues until 3 to 4 days after treatment (Figure 6). At this time there was a sudden increase in ascorbate activity in treated tissue which continued to rise throughout the testing period. The oxidation of ascorbic acid by corn tissue will be discussed in detail in Section IV of this dissertation and evidence will be offered for its nonenzymatic nature.

An attempt was made to measure cytochrome oxidase activity using a manometric method. This method involved the use of oxidized cytochrome c and a reducing agent, p-phenylenediamine. It was found that there was just as much oxygen taken up when p-phenylenediamine was used alone as there was when it was used in combination with oxidized cytochrome c. Thus, it was concluded that there was no cytochrome oxidase activity. It should be pointed out that the use of manometric techniques for cytochrome oxidase studies has been sharply criticized by Hill and Hartree (1953).

Carbon monoxide has been used to distinguish between respiration involving cytochrome oxidase and that involving the copper enzymes, phenol oxidase, and ascorbic acid oxidase. The cytochrome oxidase - carbon monoxide complex formed by this treatment is stable only in the dark and

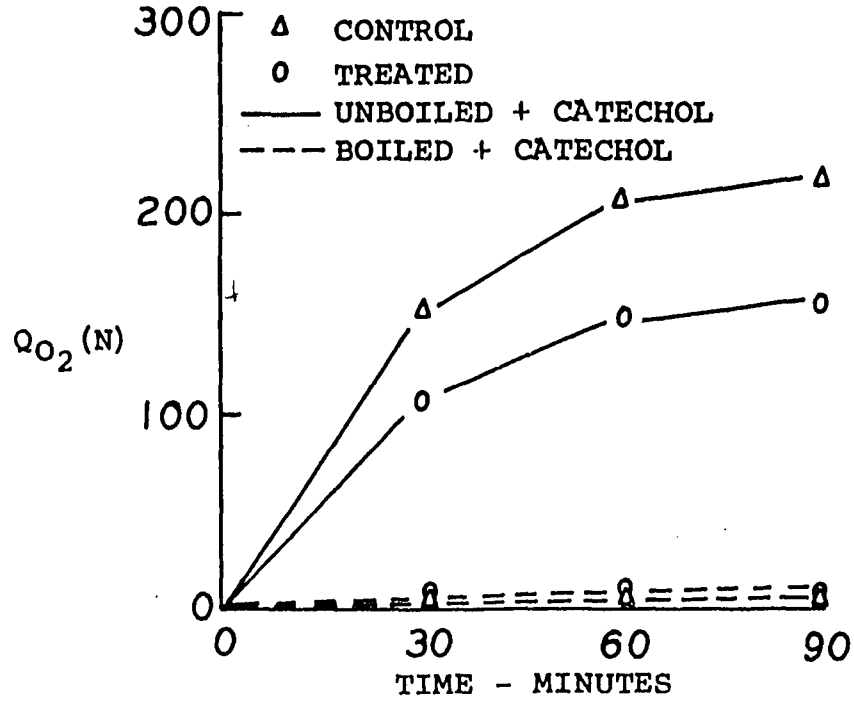


Figure 7. The effect of boiling on the activity of phenol oxidase (2 days after treatment).

is dissociated in visible light. It was found that carbon monoxide caused approximately 40 to 50 per cent inhibition of respiration (Figure 8). It was further found that light was not effective in reversing this inhibition. This adds additional evidence to the manometric data that there was no cytochrome oxidase activity.

The next respiratory enzyme studied was catalase which decomposes hydrogen peroxide to water and oxygen (Bonner, 1950). The activity of catalase was measured 2 and 6 days after dicryl treatment. It was found that catalase was very active in corn and that there was hardly any effect of dicryl on its activity 2 days after treatment. Six days after treatment, however, dicryl had caused a 50 per cent reduction in the activity of this enzyme (Figure 9A).

Peroxidase activity was found to be affected essentially the same as catalase due to dicryl treatment (Figure 10A). The per cent reduction of peroxidase activity was approximately 40 as compared to 50 with catalase. Bingham (1960) reported that on a nitrogen basis dicryl affected these two enzymes approximately the same way in cotton.

Since catalase and peroxidase are metalloprotein enzymes which contain hematin as their prosthetic groups, they should be inhibited by cyanide (Bonner, 1950). The use of 1mM KCN resulted in complete inhibition of both enzymes (Figures 9B and 10B).

The last respiratory enzyme studied was glycolic acid oxidase. According to Zelitch (1953), this enzyme reacts with glycolic acid and oxygen to form glyoxylic acid and hydrogen peroxide. This enzyme appeared to be moderately active in young corn tissue (Figure 11A). There appeared to be very little effect of dicryl on the activity of this enzyme 2 days after treatment; however, 6 days after treatment the herbicide had caused a 36 per cent reduction in activity. This finding differed significantly from

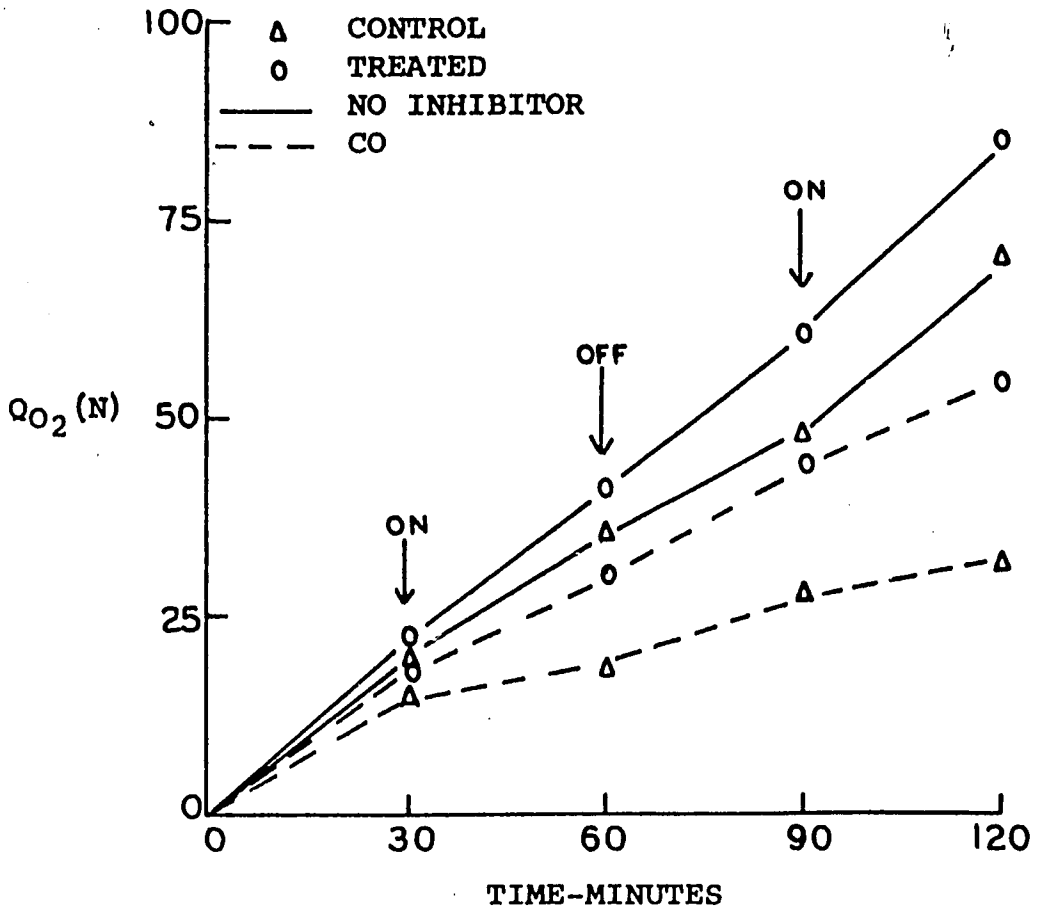


Figure 8. The effect of carbon monoxide and light on the respiration of control and treated corn tissue (2 days after treatment).

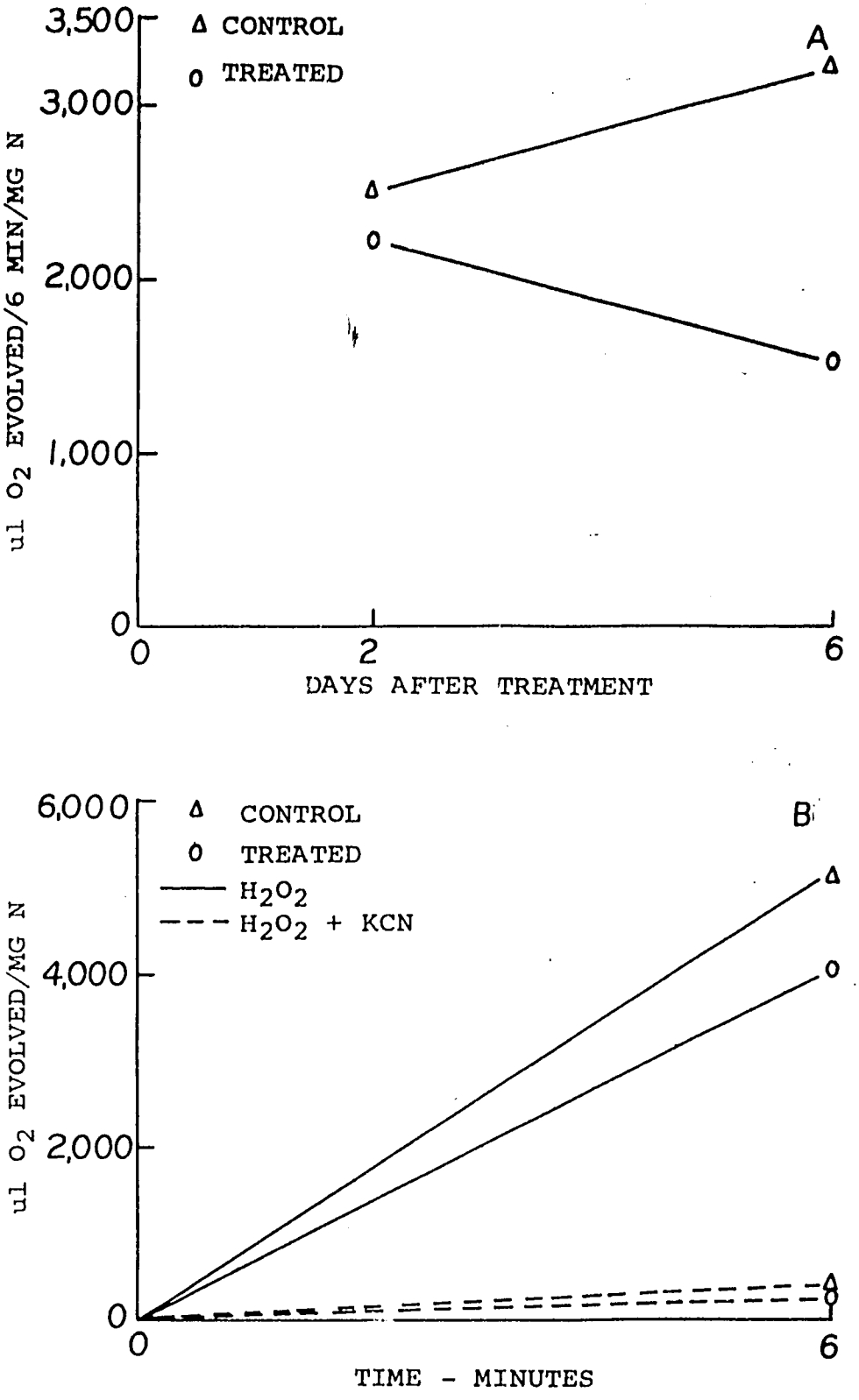


Figure 9. A. Catalase activity of corn tissue 2 and 6 days after treatment with dicryl. B. The inhibition of catalase by 1mM KCN (2 days after treatment).

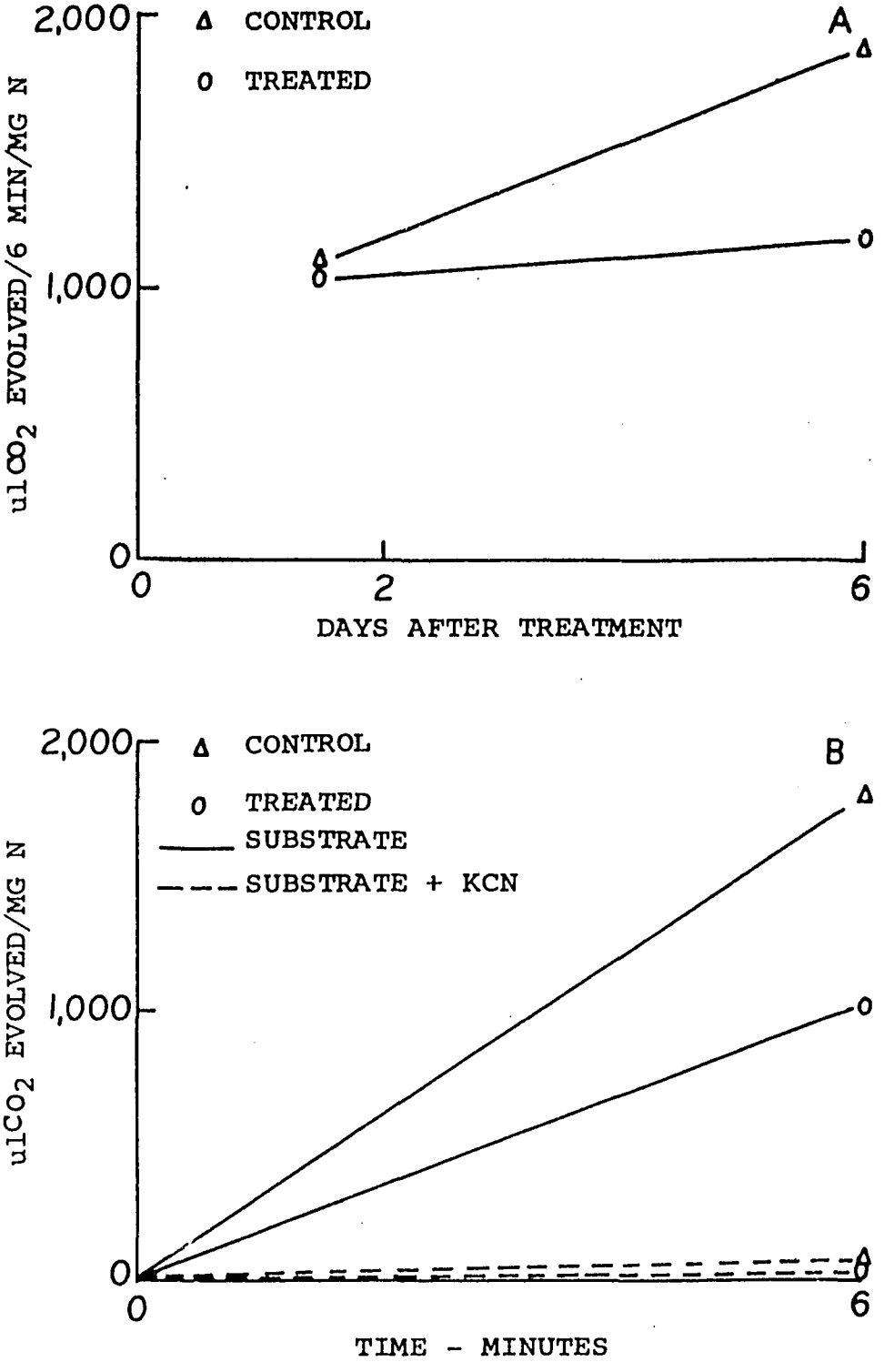


Figure 10. A. Peroxidase activity of corn tissue 2 and 6 days after treatment with dicryl. B. The inhibition of peroxidase by 1mM KCN (6 days after treatment).

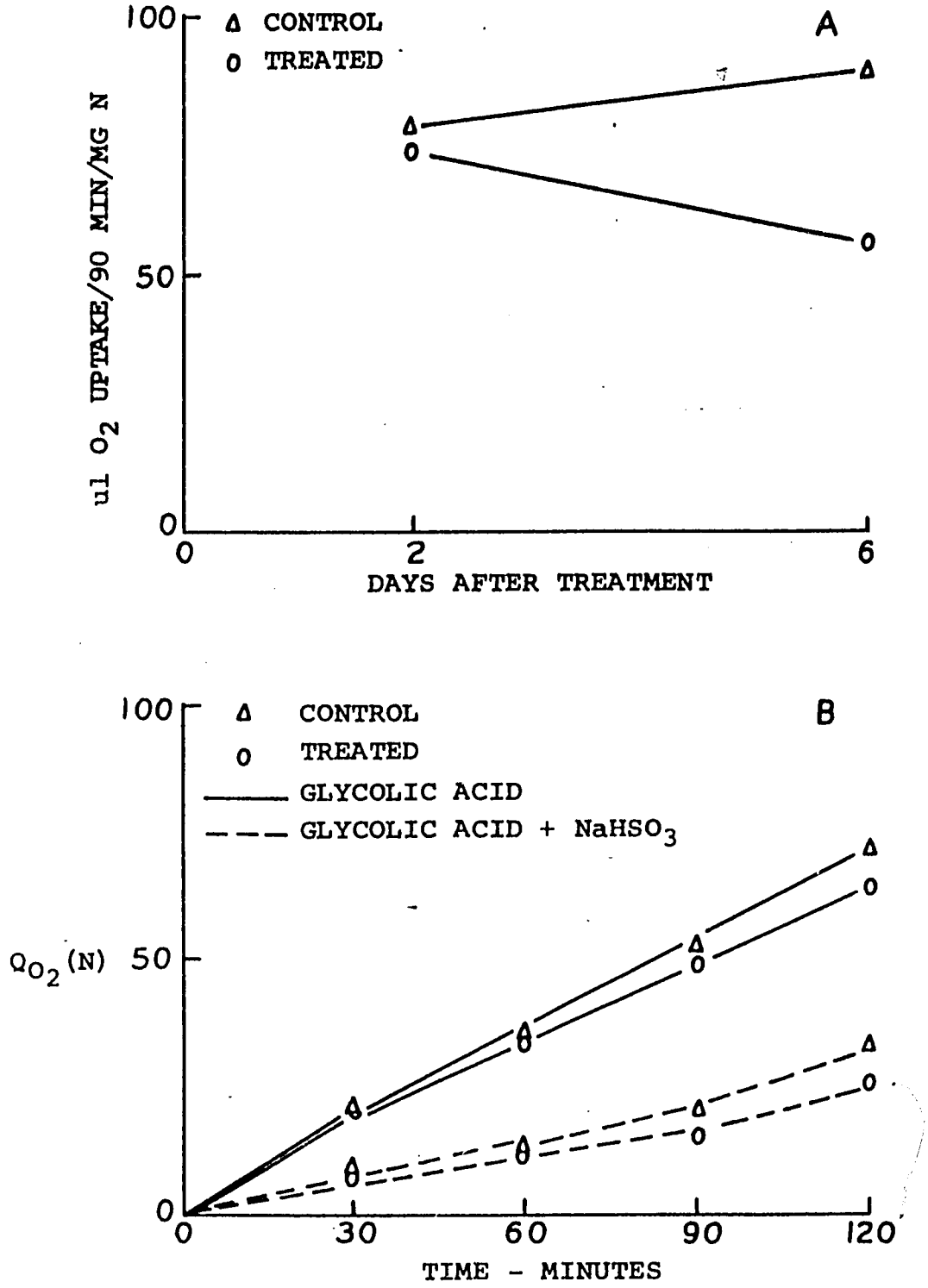


Figure 11. A. Glycolic acid oxidase activity of corn tissue 2 and 6 days after treatment with dicryl. B. The inhibition of glycolic acid oxidase by 1mM $NaHSO_3$ (2 days after treatment).

Bingham (1960) who found that dicryl did not affect the activity of glycolic acid oxidase in the cotton plant.

Zelitch (1955) reported that alpha hydroxysulfonate compounds such as acetaldehyde-bisulfite, glyoxylate-bisulfite and bisulfite alone were about equally effective in the inhibition of glycolic acid oxidase. It was found that 1 mM $NaHSO_3$ inhibited the activity of this enzyme approximately 60 per cent (Figure 11B).

IV. The Oxidation of Ascorbic Acid by Control and Dicryl Treated Corn Tissues

The increased oxidation of ascorbate by corn homogenates approximately 4 days after treatment with dicryl has been mentioned previously (Section III, Figure 6). Since this appeared to be a most significant finding it was decided the reaction should be pursued further.

A. The Effect of Boiling on the Oxidation of Ascorbate

The addition of ascorbate to homogenates is considered a common technique for determining ascorbic acid oxidase activity. Ascorbate, in addition to being oxidized by ascorbic acid oxidase, can be oxidized by phenol oxidase, laccase, cytochrome oxidase, and peroxidase (Mapson, 1958). In order to check on the nature of the substance or substances responsible for oxidizing ascorbate, it was decided to measure the effect of boiling on the reaction. It was found that boiling for 2 minutes slightly increased the oxidation of ascorbate by both control and treated homogenates (Figure 12B). This indicated the substance responsible for the oxidation of ascorbate was not an enzyme.

B. The Effect of Copper on the Oxidation of Ascorbate

At this stage of the investigation the oxidation of ascorbate was suspected to be of a non-enzymatic nature and that ions, possibly copper, were the cause of oxidation. It was found that 1 ppm $CuSO_4$ caused only a

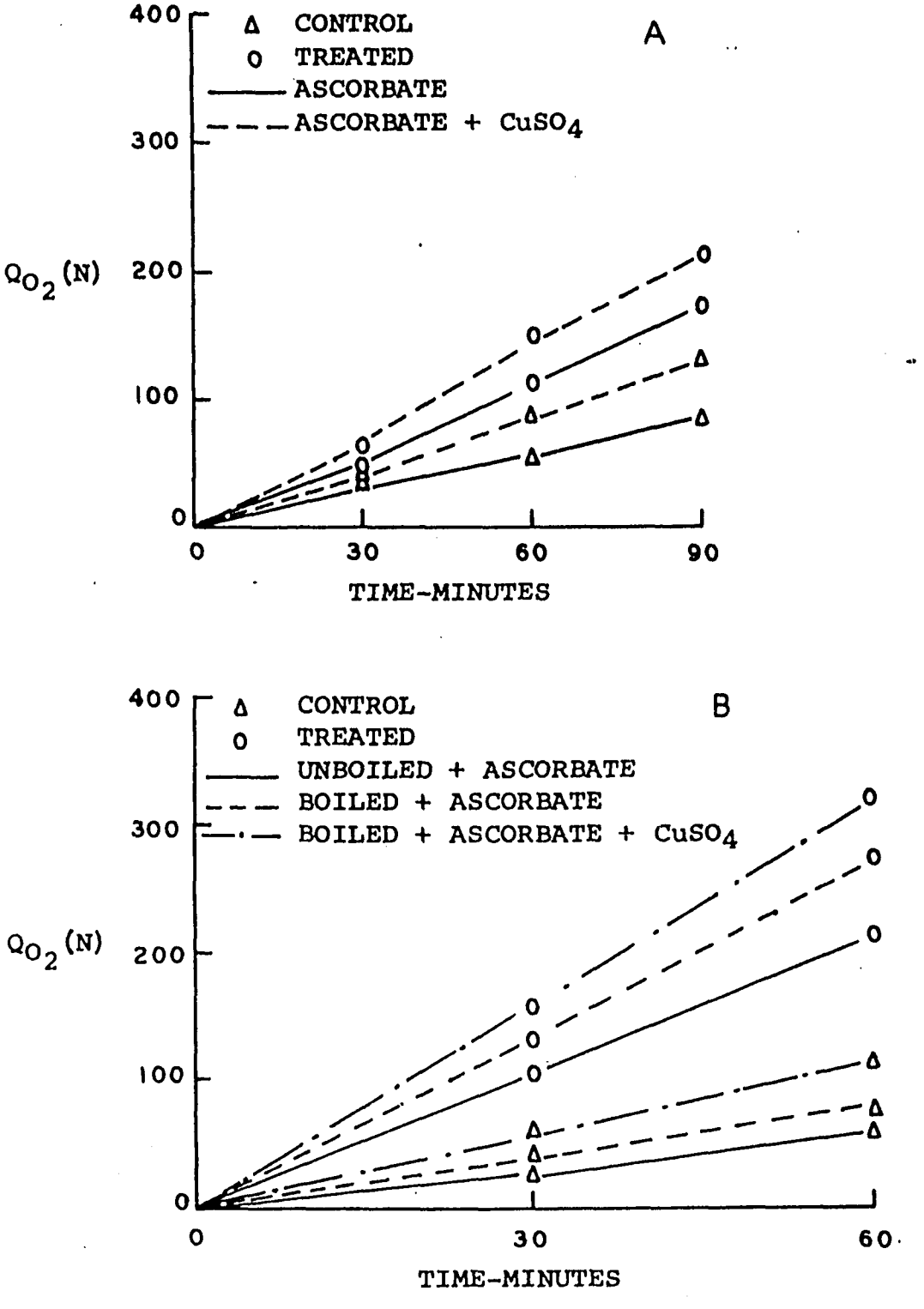


Figure 12. A. The influence of copper (1ppm) on the oxidation of ascorbic acid. B. The influence of boiling and copper on the oxidation of ascorbic acid (5 days after dicryl treatment).

slight increase in the oxidation of ascorbate in both control and treated homogenates (Figure 12A). Copper also caused a slight increase in oxidation of ascorbate in homogenates which had been previously boiled (Figure 12B). The increase in oxidation of ascorbate by homogenates due to copper was slight compared to that by various inorganic systems (Table 1).

C. The Results of Heavy Metal Inhibitors and a Cation Exchange Resin on the Oxidation of Ascorbate

A series of heavy metal inhibitors were utilized to further measure the influence of ions on the oxidation of ascorbate. Potassium cyanide, sodium diethyldithiocarbamate (dieca), 8-hydroxyquinoline, potassium ethyl xanthate, and salicylaldoxime were all used at several concentrations (0.2 to 10 mM) as possible inhibitors. None of these materials caused any significant reduction in the oxidation of ascorbate. McWhorter (1958) found that dieca reduced the oxidation of ascorbate in corn tissue by only a very small amount.

A cation exchange resin (Amberlite IR 120) was used in an attempt to remove any cations which might influence the oxidation of ascorbate. It was found after supernatants (1,000 xg) were treated with Amberlite that there was very little change in the oxidation of ascorbate (Figure 13B). The effeciency of the cation exchange resin can be determined from the data illustrated in Figure 13A.

It was found that $CuSO_4$ had the same effect on the oxidation of ascorbate in supernatants which had not been treated with Amberlite as it did in those which had been treated with the cation exchange resin (Figure 14). It was concluded at this stage of the investigation that the oxidation of ascorbate by corn tissue was not due to an enzyme nor did it appear to be catalyzed by metals.

Table I The effect of copper on the oxygen uptake of inorganic systems (pH 5.85) containing ascorbate and catechol.

	Inorganic System	ul O_2 uptake after 30 minutes
1	0.5 ml ascorbate + 2 ml KH_2PO_4 buffer	13.5
2	0.5 ml ascorbate + 2 ml KH_2PO_4 buffer + 1 ppm $CuSO_4$	240.0
3	0.5 ml ascorbate + 2 ml glass distilled H_2O	1.2
4	0.5 ml catechol + 2 ml KH_2PO_4 buffer	0.0
5	0.5 ml catechol + 2 ml KH_2PO_4 buffer + 1 ppm $CuSO_4$	2.0
6	0.5 ml catechol + 2 ml glass distilled H_2O	1.2

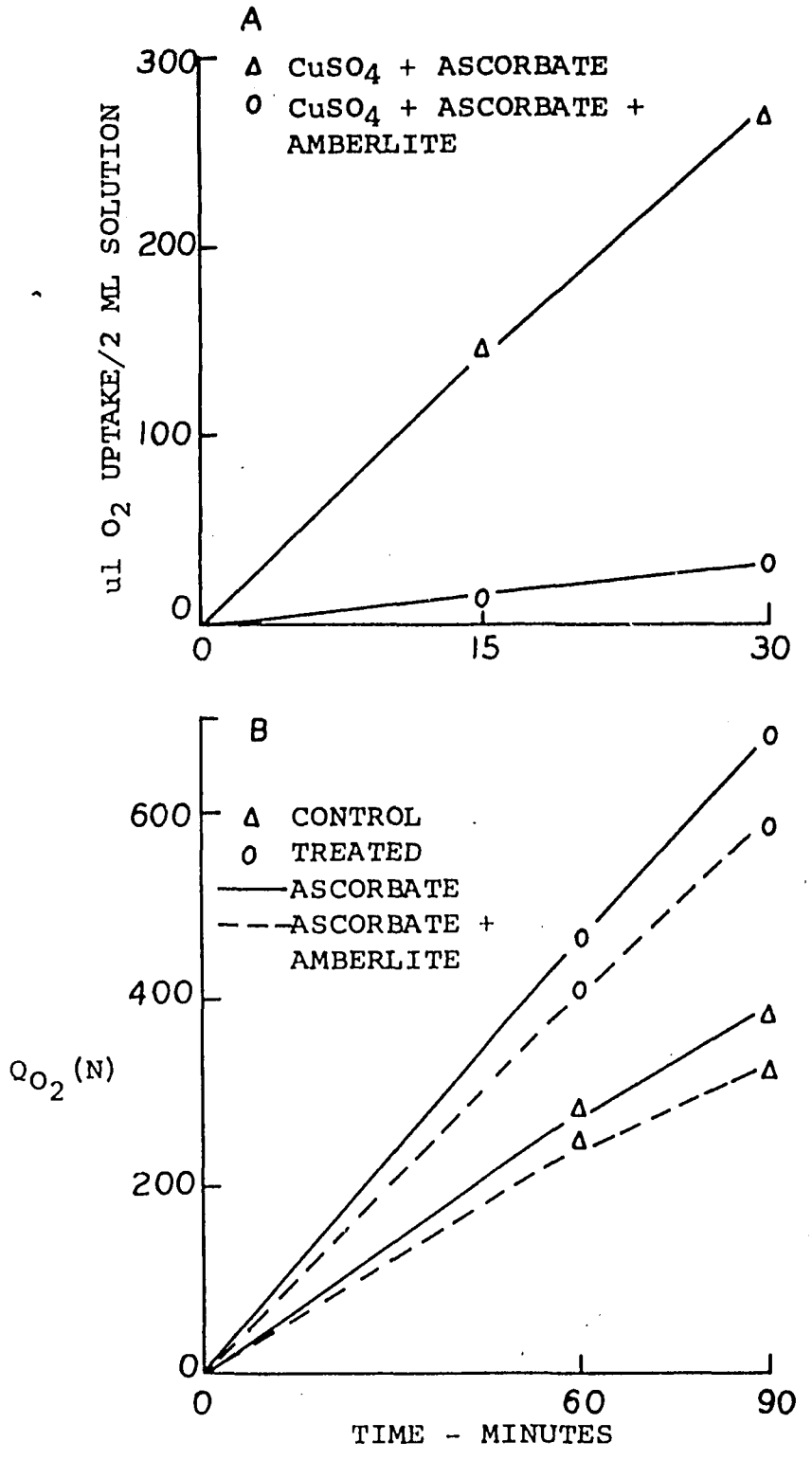


Figure 13. A. The influence of a cation exchange resin (Amberlite IR 120) on copper catalyzed (nonenzymatic) oxidation of ascorbic acid. B. The influence of a cation exchange resin on the oxidation of ascorbic acid in supernatants (1,000 xg) from control and treated corn tissue.

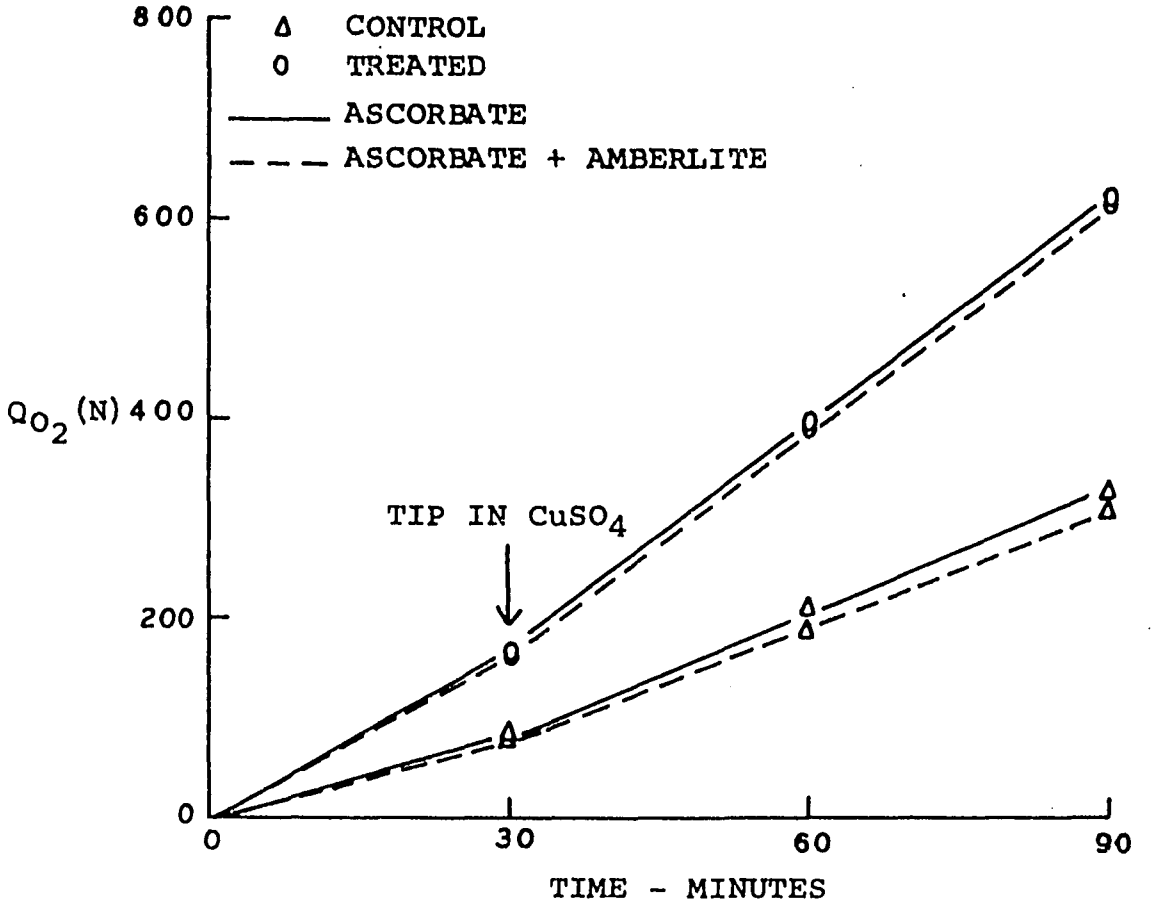


Figure 14. The effect of copper (1 ppm) on the oxidation of ascorbic acid by supernatants (1,000 xg) which had been treated with a cation exchange resin.

D. Investigation of the Possibility of Ascorbate as a Source of Electrons for the Reduction of Quinones

This possibility was measured 2 and 5 days after dicryl treatment. In both cases it was shown that the oxidation of ascorbic acid and phenol oxidase activity were additive (Figures 15 and 16).

It was noticed when the oxidation of ascorbate began to increase in treated tissue the plants began to show the first visible injury symptoms. The first sign of injury was a slight, general chlorosis of the leaf. The leaf then turned a yellowish color followed by a dark brown during the next 4 to 6 days. It was decided to injure the leaves by steam and then measure the oxidation of ascorbate by homogenates from this tissue. Twenty-four hours after the steam treatment it was found that ascorbate was oxidized much more rapidly by injured tissue than by control (Figure 17).

E. Dialysis Studies

The effects of dialyzing homogenates and supernatants on the oxidation of ascorbate are illustrated in Figures 18A and 19. The oxidation of ascorbate was reduced by dialysis in both control and treated tissues. All data in Figure 18A are plotted on the nitrogen content of undialyzed homogenates whereas in Figure 19 the data are plotted on the nitrogen content of each individual sample. When supernatants were dialyzed it was found that the nitrogen content of control supernatants was 30 per cent less than undialyzed and the nitrogen content of dialyzed treated supernatants was 64 per cent less than undialyzed. If the data in Figure 19 were plotted on the same basis as that in Figure 18A it would be more obvious that there was a great loss in activity due to dialysis. Since considerably more nitrogen was lost from dialyzed treated than from dialyzed control supernatants, it was concluded that the treatment with dicryl caused a breakdown of nitrogenous compounds.

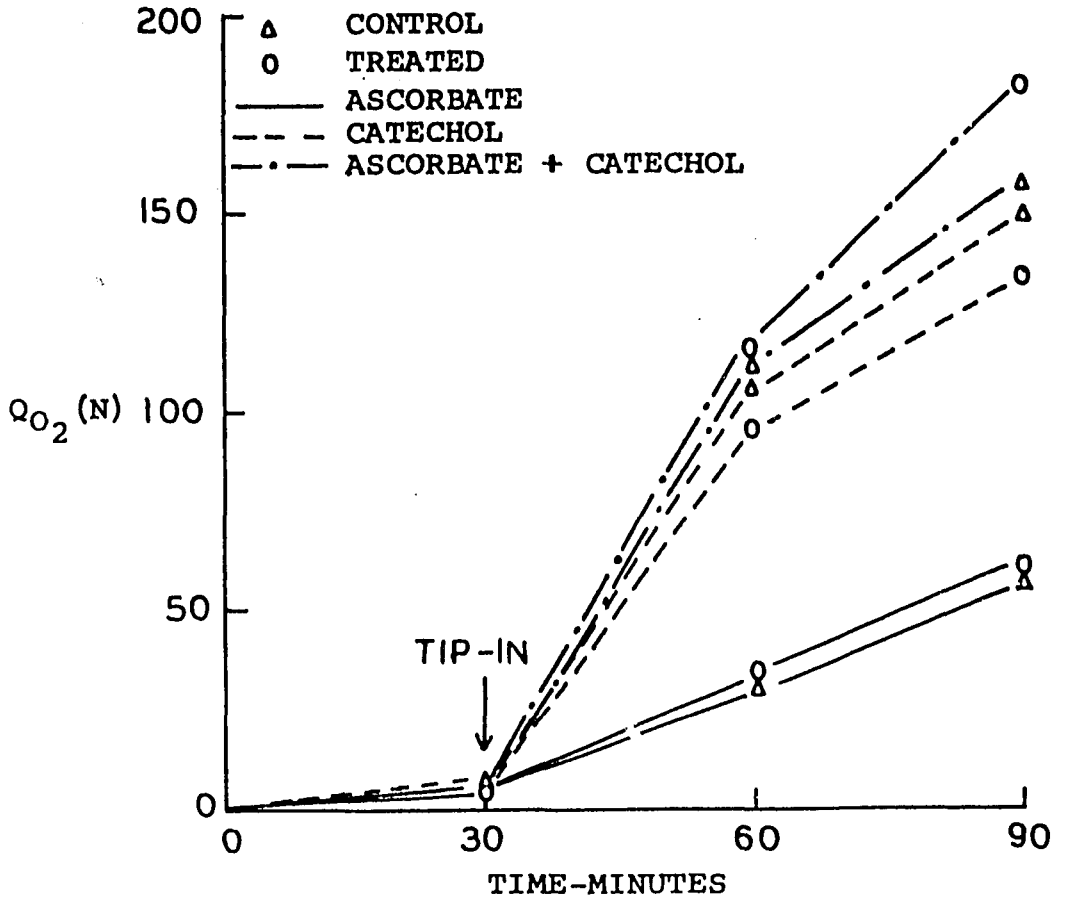


Figure 15. The effect of adding ascorbate and catechol individually and in combination to control and treated corn tissue (2 days after treatment with dicryl).

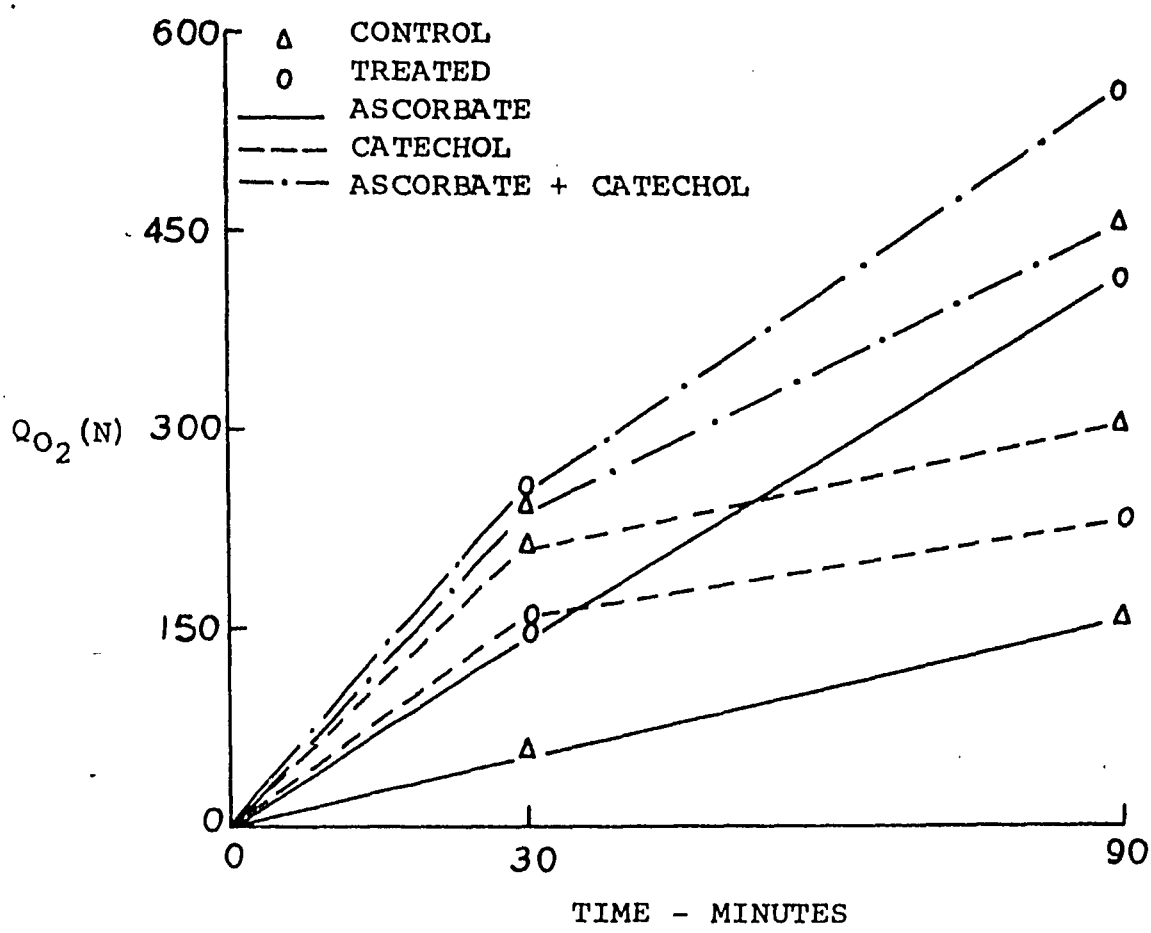


Figure 16. The effect of adding ascorbic acid and catechol individually and in combination to control and treated corn tissue (5 days after treatment with dicryl).

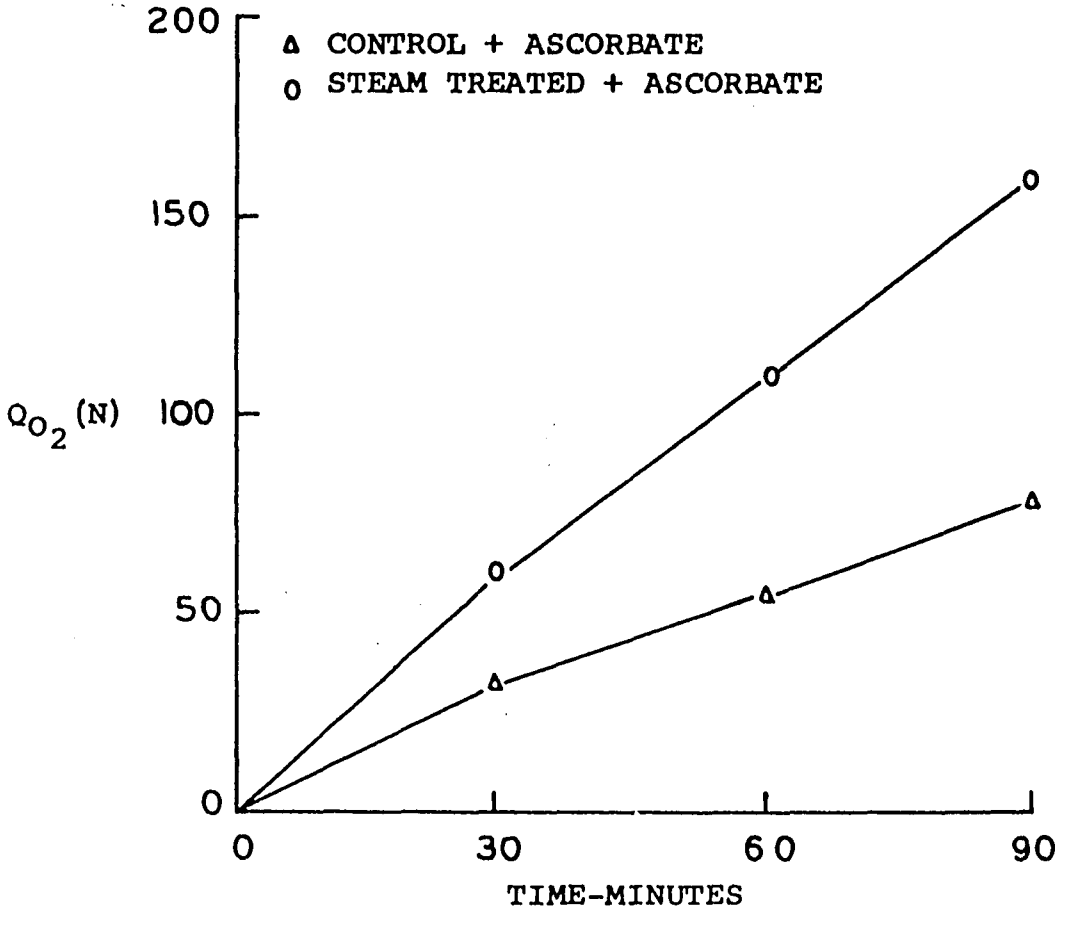


Figure 17. A comparison of ascorbic acid oxidation in steam treated and control corn tissue.

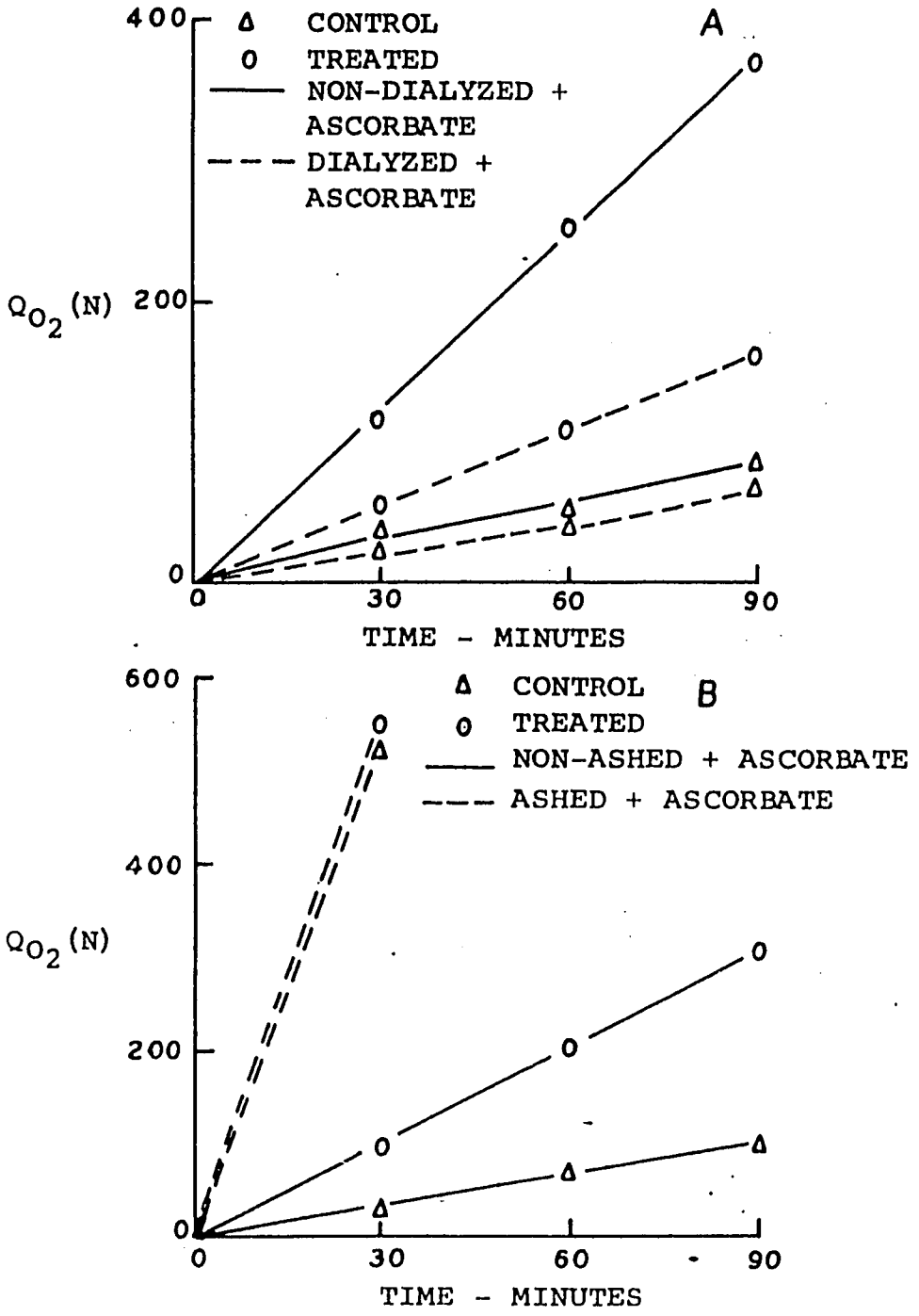


Figure 18. A. The effect of dialysis on ascorbic acid oxidation. B. A comparison of the oxidation of ascorbate by ashed and nonashed homogenates from control and dicryl treated corn tissue.

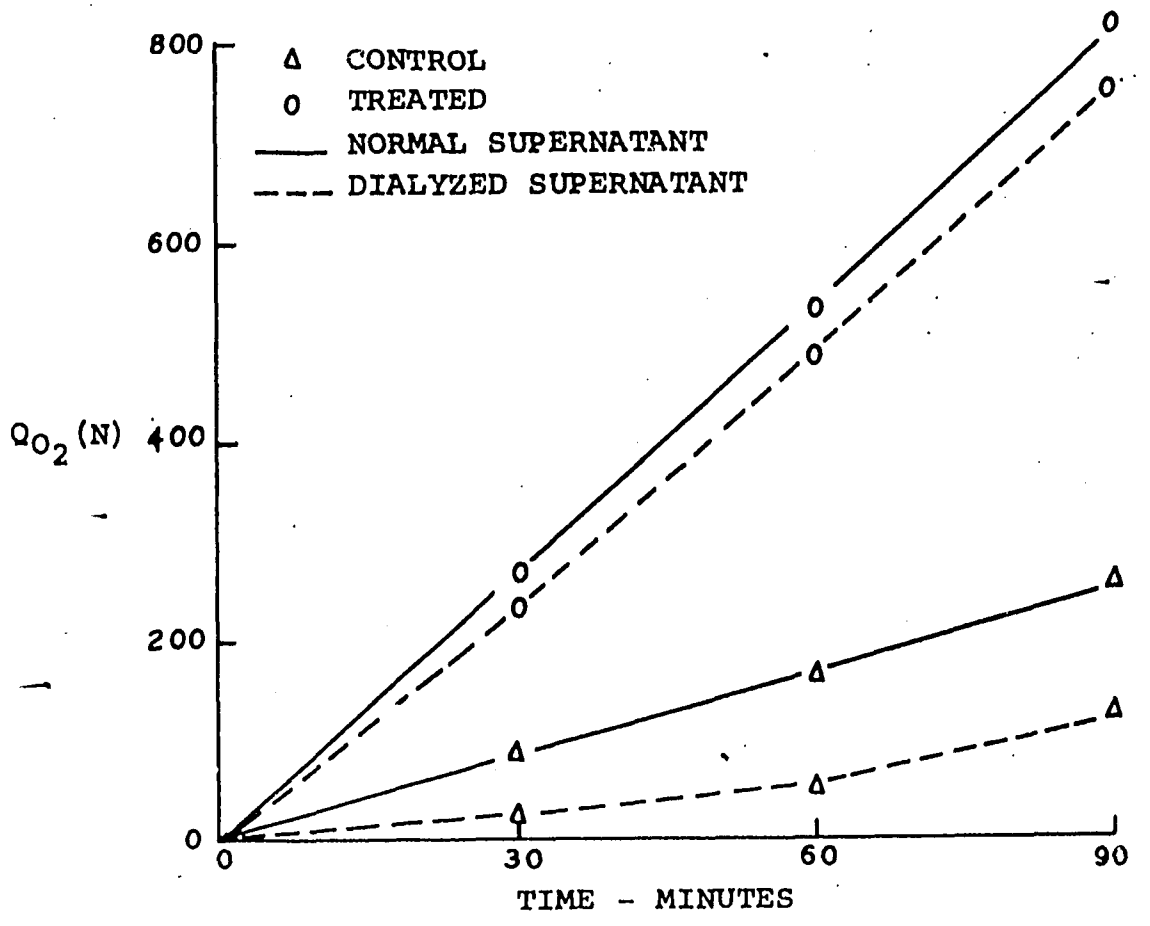


Figure 19. The effect of dialysis on the oxidation of ascorbic acid. (The N content of dialyzed control supernatant was 30 per cent less than nondialyzed and the N content of dialyzed treated supernatant was 64 per cent less than nondialyzed. The data are plotted on the N content of each sample).

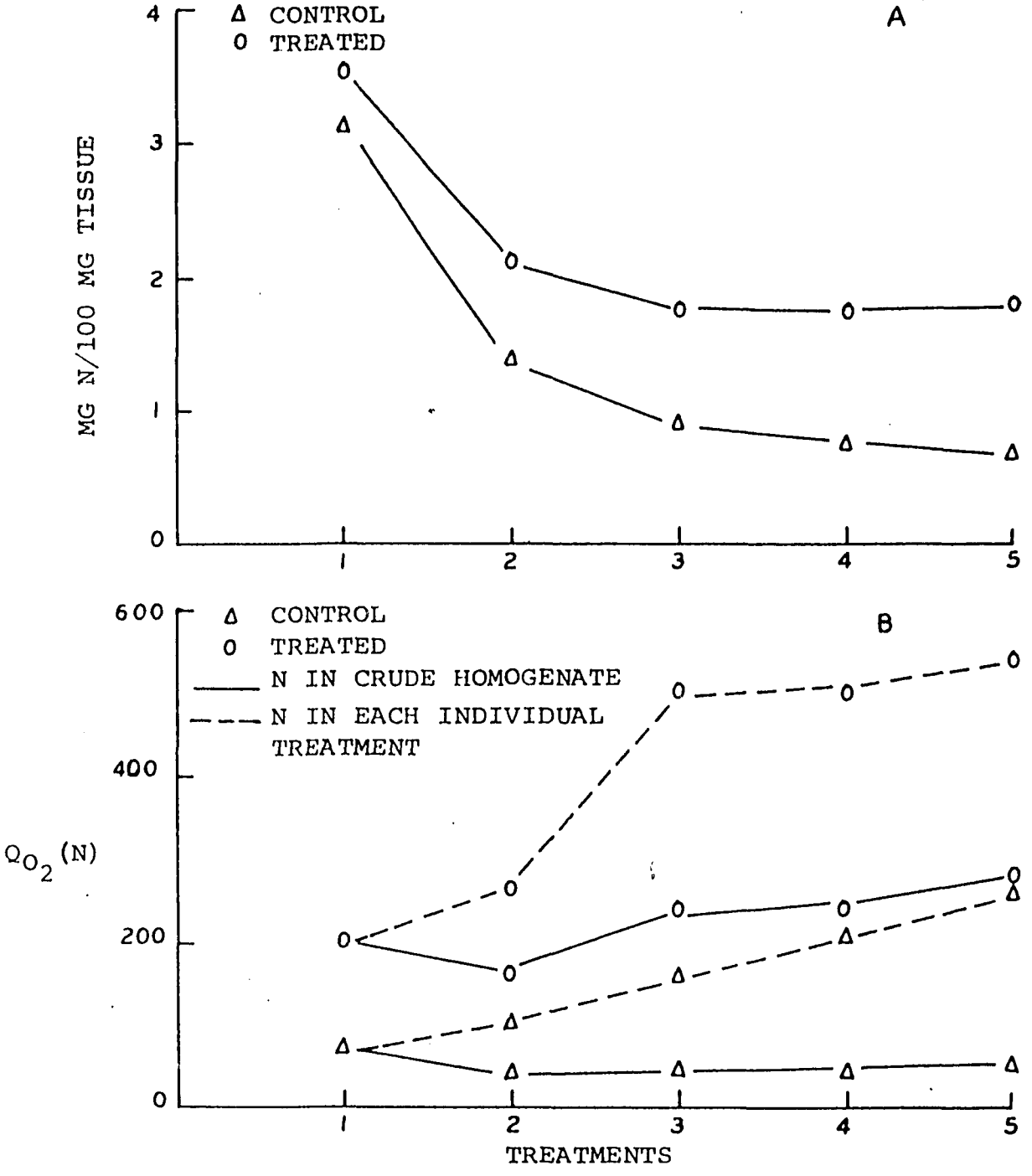
F. Ashing of Homogenates

It was decided to measure the oxidation of ascorbic acid by homogenates which had been ashed. It was assumed that the process of heating the homogenates for 2 hours in a crucible over a Bunsen burner did away with all organized forms of proteins. After the homogenates had been ashed, it was found that they very rapidly oxidized ascorbic acid (Figure 18B). The oxidation of ascorbate by ashed homogenates was considered to be nonenzymatic. This oxidation was thought to be due entirely to ions, probably copper, which were in the tissue.

G. Fractionation by Centrifigation

Supernatants from homogenates, which were subjected to various speeds of centrifugation (1,000-144,000 xg), were checked for the oxidation of ascorbate. The influence of centrifugation on nitrogen content of the various supernatants was also determined. In whole homogenates the nitrogen content of treated tissue was only 10 per cent more than that in control tissue (Figure 20A). After centrifugation at 144,000 xg there was 74 per cent more nitrogen in treated supernatants than in control. This adds additional evidence to dialysis experiments in support of the conclusion that proteins are broken down into smaller components due to dicryl treatment.

The most significant fact brought out by this experiment was that the oxidation of ascorbate was not appreciably influenced by centrifugation (Figure 20B). The data in this graph are plotted on the nitrogen content of the crude homogenate and on the nitrogen content of each individual sample. On a nitrogen basis, it is obvious that as the nitrogen content of the sample goes down due to centrifugation the activity would automatically go up. In considering the activity on a volume basis, 1 ml of crude homogenate contained approximately the same activity as 1 ml



TREATMENTS:
1 - Crude homogenate
2 - Supernatant (1000 x g)
3 - Supernatant (48,000 x g)
4 - Supernatant (96,000 x g)
5 - Supernatant (144,000 x g)

Figure 20. **A. The influence of centrifugation on the N content of various samples. B. The influence of centrifugation on the oxidation of ascorbic acid. Data are plotted on N content in the crude homogenate and on the N content in each individual sample.**

of any supernatant (1,000-144,000 xg).

At this stage of the investigation it was concluded that there was some substrate in the corn tissue which was responsible for the oxidation of ascorbate. This substrate was probably a relatively small compound since it moved through a dialysing membrane and it remained in the supernatant after high speeds of centrifugation.

H. Possible Substitutes for Ascorbate

The role of ascorbate in hydroxylation of aromatic compounds within a model system has been studied by Udenfriend et al. (1954) and Brodie et al. (1954). In their studies they found that dehydroascorbate would substitute for ascorbate below pH 6.5. This possibility was checked in corn tissue. There was no oxygen uptake when dehydroascorbate was added to control or treated homogenates of corn tissue (Figure 21A).

La Du (1953) reported that in animal tissue hydroquinone could replace ascorbate in vitro but not in vivo. This possibility was checked (in vitro) for corn tissue. The oxygen uptake which resulted from the addition of hydroquinone to control and treated homogenates was very small (Figure 21B). It appeared that neither hydroquinone nor dehydroascorbate could substitute for ascorbate in corn tissue.

I. Cucumber Ascorbic Acid Oxidase

At this stage of the investigation it appeared that the reaction of ascorbate with control and dicryl treated corn tissue was not a usual one. In order to check some of the techniques which had been utilized thus far in the study it was decided to extract a known source of the enzyme ascorbic acid oxidase (AAO). Cucumber was selected since it was reported to contain a very active source of the enzyme (Snow and Zilva, 1938) and because it was readily available.

Cucumber extract oxidized ascorbic acid approximately 14 times

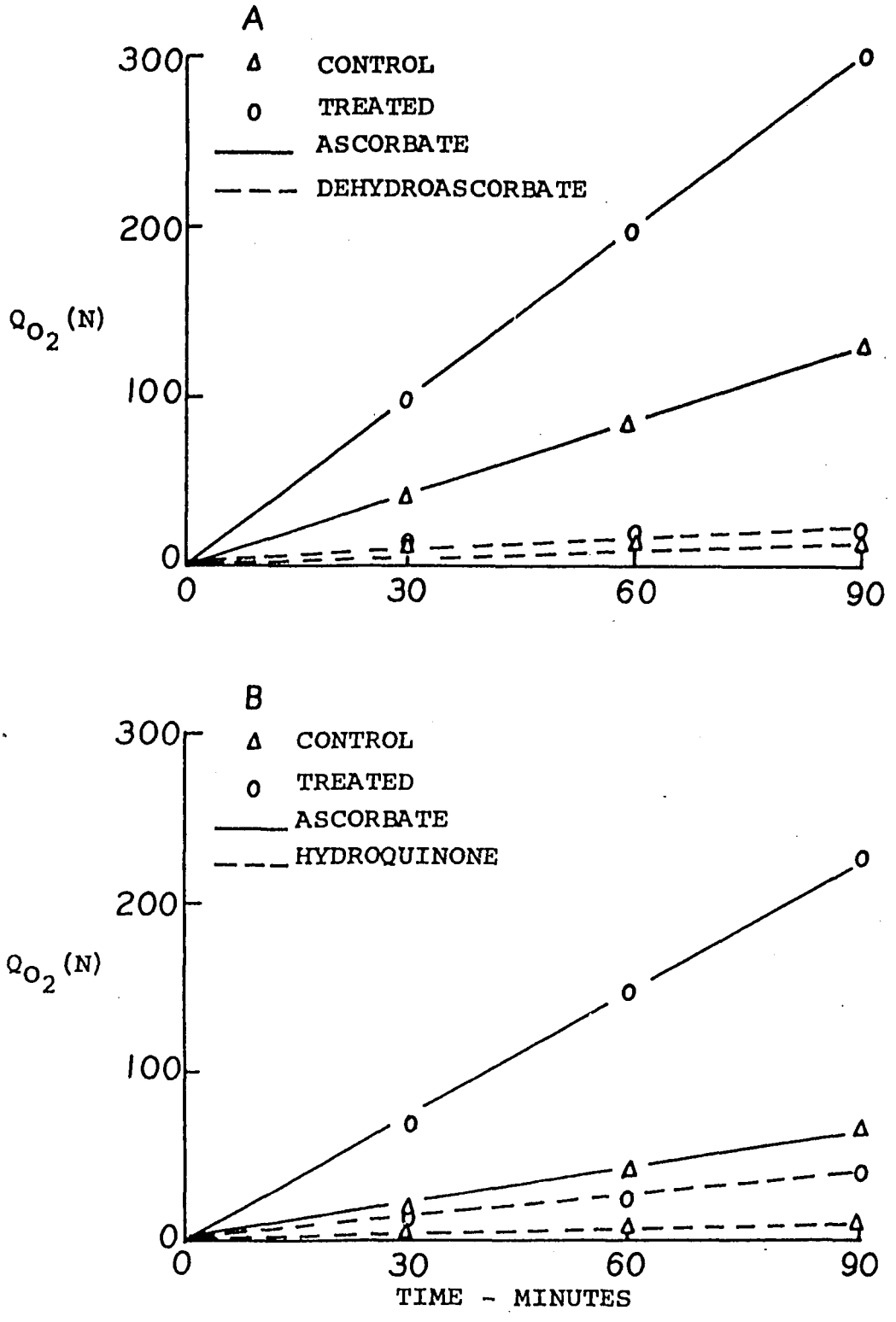


Figure 21. A. A comparison of oxygen uptake in control and dicryl treated corn tissue when (A) ascorbic and dehydroascorbic acid and (B) ascorbic acid and hydroquinone were added as substrates.

faster than treated and 60 times faster than control homogenates of corn (Figure 22). In the same graph it can be seen that boiling stopped completely the oxidation of ascorbate by cucumber extract while the oxidation of ascorbate by control and dicryl treated corn homogenates was slightly increased. In this same experiment it was decided to see what effect boiling would have on combinations of corn homogenates and cucumber extract. From the data illustrated in Figure 22, it is evident that boiling inactivated the enzyme in cucumber while corn homogenates (control and treated) retained their ability to oxidize ascorbic acid.

The effect of unboiled combinations of cucumber extract and corn homogenates can be seen in Figure 23A. The effect of adding the substrates together appeared to be additive. This experiment was performed to check the possibility of inhibitors of the oxidation of ascorbate such as those mentioned earlier (Damordaron and Nair, 1936, Hooper and Ayres, 1950, and Jackson and Wood, 1959). From these data it would have to be concluded that there was nothing in corn homogenates which affected the oxidation of ascorbate in cucumber extract and vice versa.

The next experiment involved measuring the influence of dieca on the oxidation of ascorbate by cucumber extract. Complete inhibition of the cucumber enzyme was obtained with 0.2 mM dieca (Figure 23B). The effect of dieca on the oxidation of ascorbate by corn homogenates can once again be seen in the same figure. It also seemed desirable to measure the effect of dieca on combinations of cucumber extract and corn homogenates. In these combinations dieca completely inhibited the cucumber enzyme while the oxidation of ascorbate by corn homogenates was unaffected.

At this stage of the investigation it should be emphasized that the substance which catalyzed the oxidation of ascorbate in cucumber extract

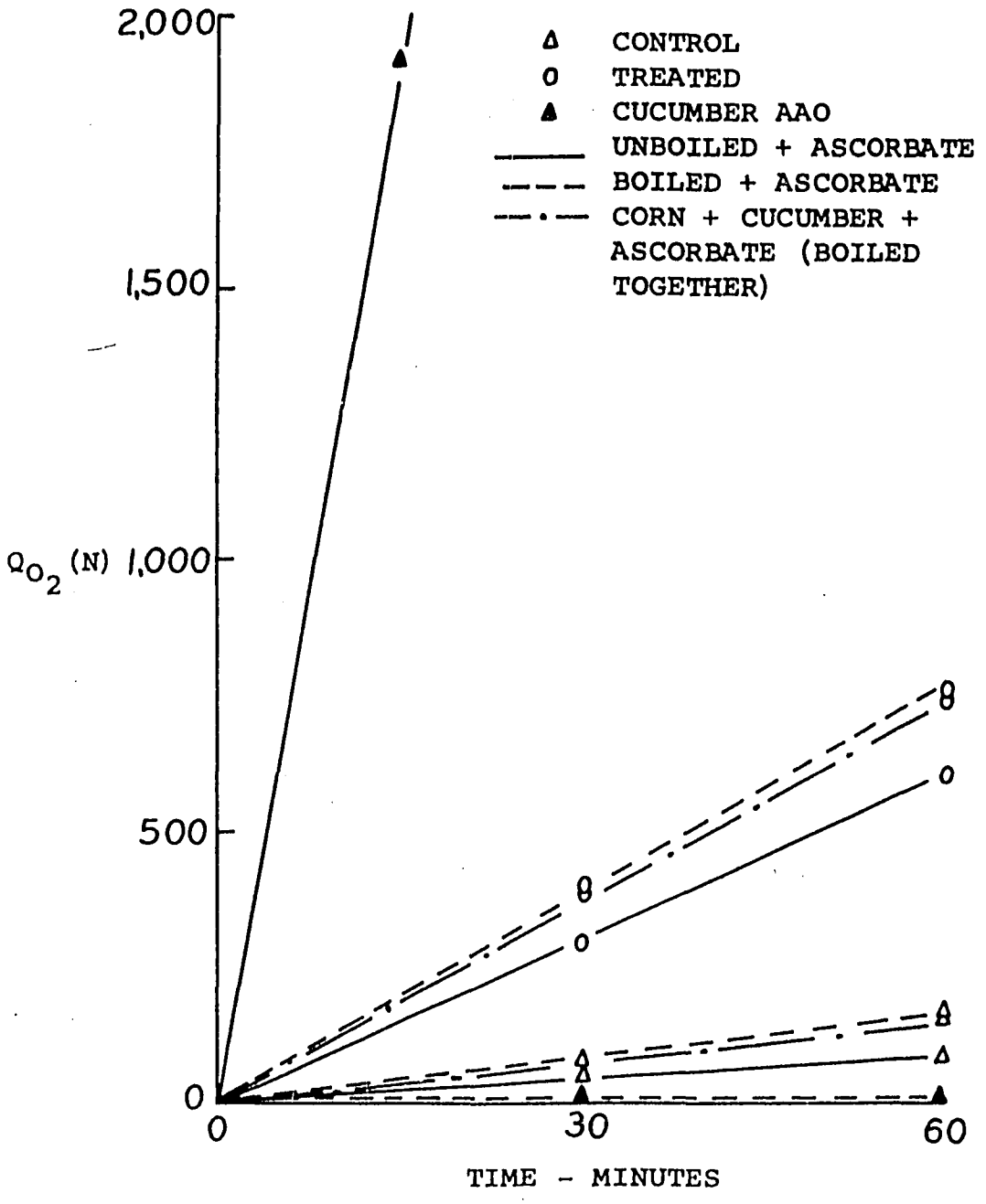


Figure 22. The effect of boiling on the oxidation of ascorbic acid by corn and cucumber extracts. The extracts were used individually and in combination.

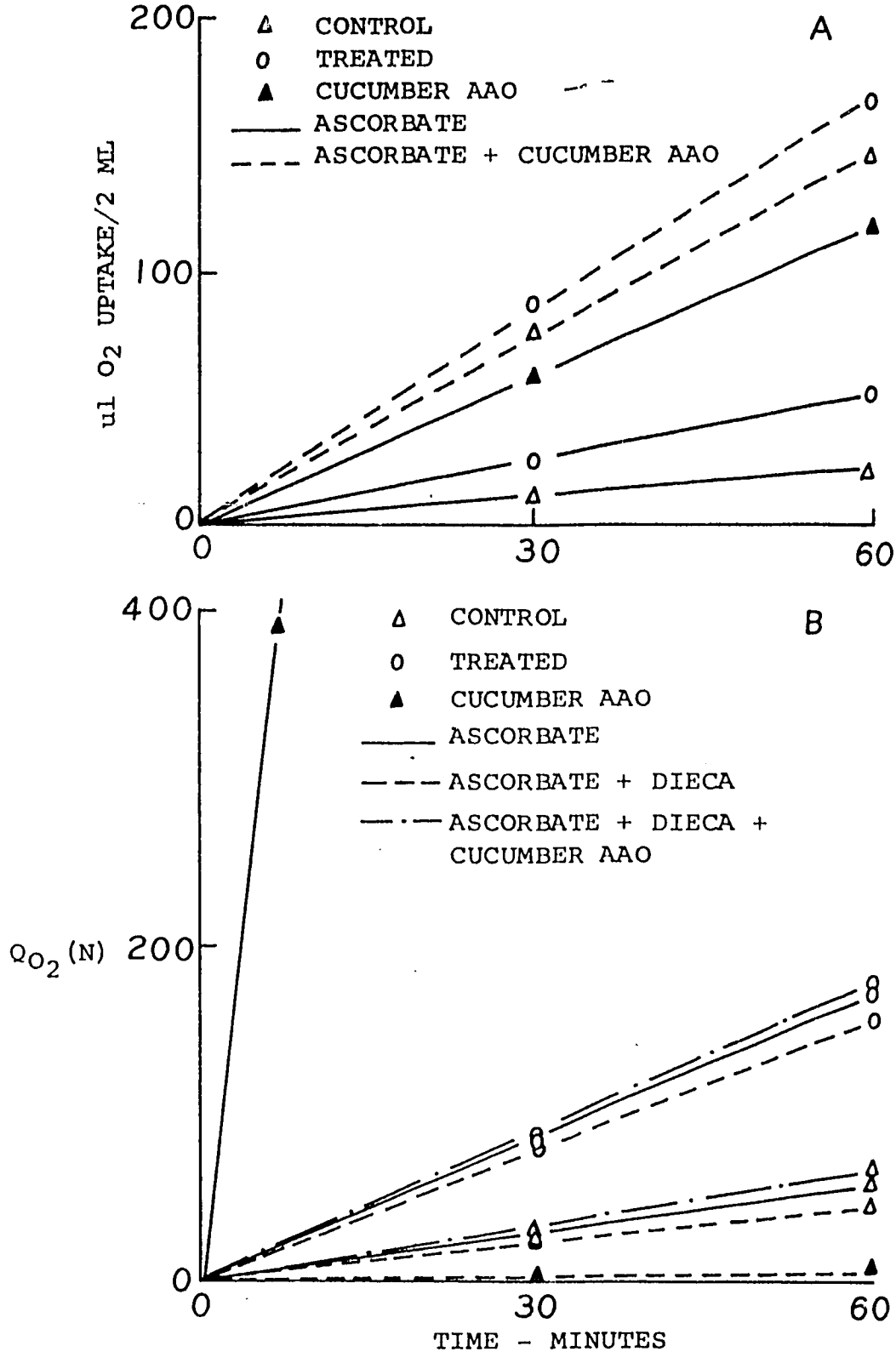


Figure 23. A. The oxidation of ascorbic acid by control and dicryl treated corn homogenates, cucumber extract, and combinations of these. B. The influence of dieca on the oxidation of ascorbic acid by the same systems as mentioned in A.

meets some of the most critical requirements of an enzyme. These are that the substance was completely inactivated upon boiling and by the addition of an inhibitor. In addition, it should be re-emphasized that these two treatments had no inhibitory effect upon the oxidation of ascorbate by corn homogenates or supernatants.

J. Paper Chromatography Studies

Since prior work had indicated nitrogenous materials were broken down in treated tissues, it was decided to determine the identity of some of these compounds. Through the use of one and two dimensional chromatography it was found that treated tissue contained considerably more alanine, asparagine, and glutamine than control tissue. The Rf values of these compounds in a butanol - acetic acid - water solvent were 0.37, 0.13, and 0.17 respectively, and in a collidine - lutidine - water solvent they were 0.17, 0.09, and 0.13 respectively.

The intensity of the color reaction of the amino acids with ninhydrin was compared between control and treated tissue in five different tests. It was found that control tissue contained 24 per cent as much asparagine, 42 per cent as much glutamine, and 55 per cent as much alanine as treated tissue. The treated tissue possibly contained a greater content of several other amino acids but the ones mentioned above were by far the predominant ones.

The effects of alanine, asparagine, glutamine, and phenylalanine on the oxidation of ascorbate by control homogenates were determined. Phenylalanine was added because it is an aromatic compound and could possibly be oxidized by means of hydroxylation. This reaction is known to occur in model systems (Udenfriend et al. 1954). The amino acids caused no effect at 0.1 per cent but at 0.1 M phenylalanine and alanine caused no effect while asparagine and glutamine caused considerable

inhibition (Figure 24). It was concluded from these experiments that an increase in alanine, asparagine, and glutamine was not involved in the increased oxidation of ascorbate by treated tissue.

K. The Influence of Iodoacetate on Ascorbate Oxidation

Since only metal complexers had been used, it was decided to determine the influence of a sulfhydryl inhibitor on the oxidation of ascorbate. Iodoacetate, at concentrations of 0.01, 0.05, and 0.1 M was used for this purpose. The lowest concentration of this inhibitor caused a slight stimulation of oxygen uptake whereas higher rates caused a marked reduction in oxygen uptake (Figure 25). It should be emphasized that iodoacetate inhibited the oxidation of ascorbate considerably more than any other inhibitor. It should also be emphasized that the concentrations which caused inhibition were greater than those commonly used for most inhibitors. The data from this one inhibitor indicated that the substance responsible for the oxidation of ascorbate contained sulfhydryl groups.

L. Influence of Glutathione on the Oxidation of Ascorbate

Since a sulfhydryl inhibitor indicated that possibly compounds which contained -SH groups could be responsible for the oxidation of ascorbate, the influence of glutathione on the reaction was determined. Reduced (GSH) and oxidized (GSSG) glutathione at 0.05 M was added to control and treated homogenates. It was found that neither GSH nor GSSG caused any oxygen uptake when added to these homogenates. When ascorbate was added to homogenates in the presence of glutathione, both GSH and GSSG caused a reduction in oxygen uptake (Figure 26).

The presence of -SH groups did not cause the expected increase in oxygen uptake but instead caused a significant reduction. The reduction was possibly of the same nature as that encountered with amino acids

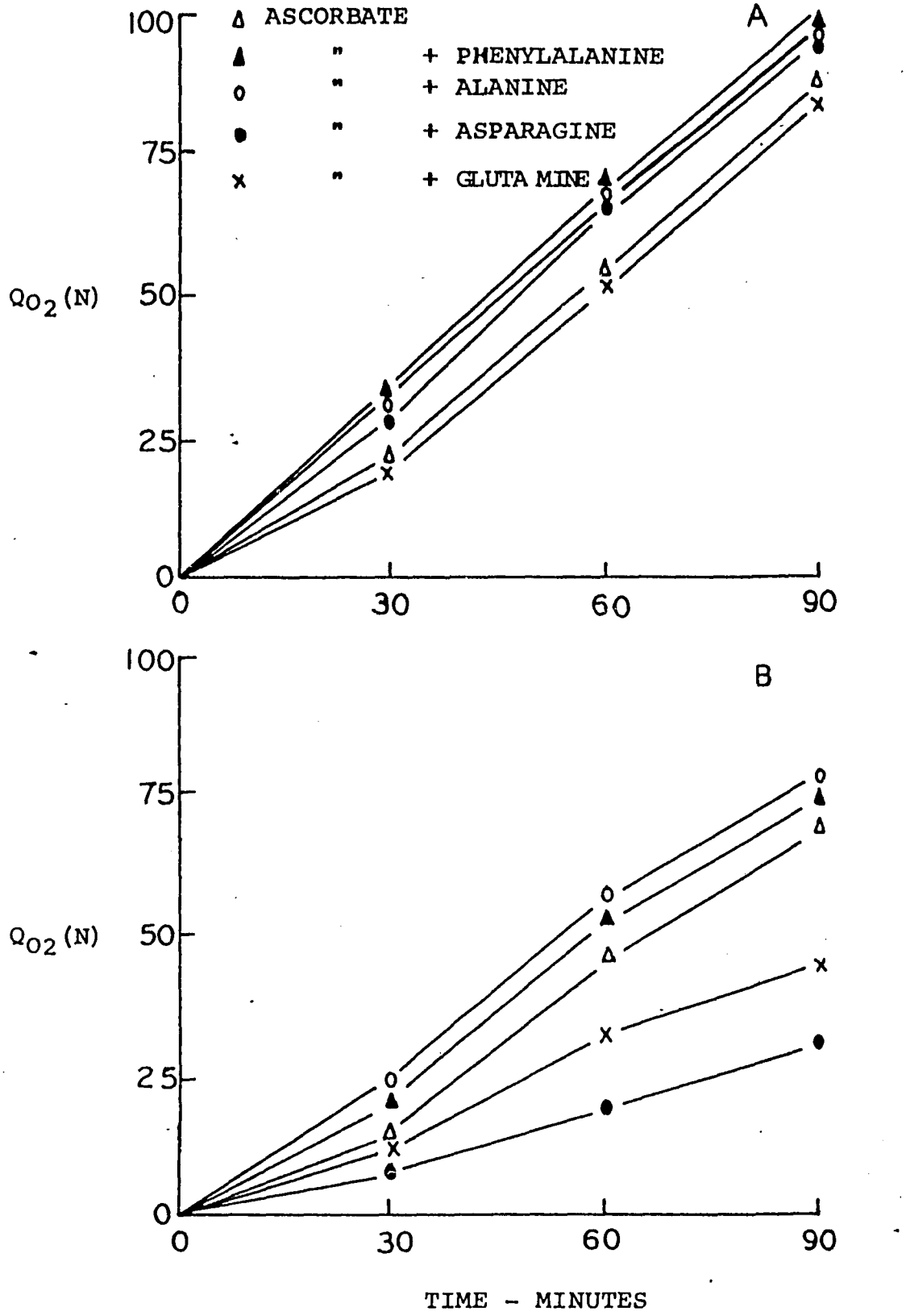


Figure 24. The influence of several amino acids on the oxidation of ascorbic acid by untreated corn homogenates. The concentration of amino acids in A was 0.1 per cent (w/v) and in B it was 0.1 M.

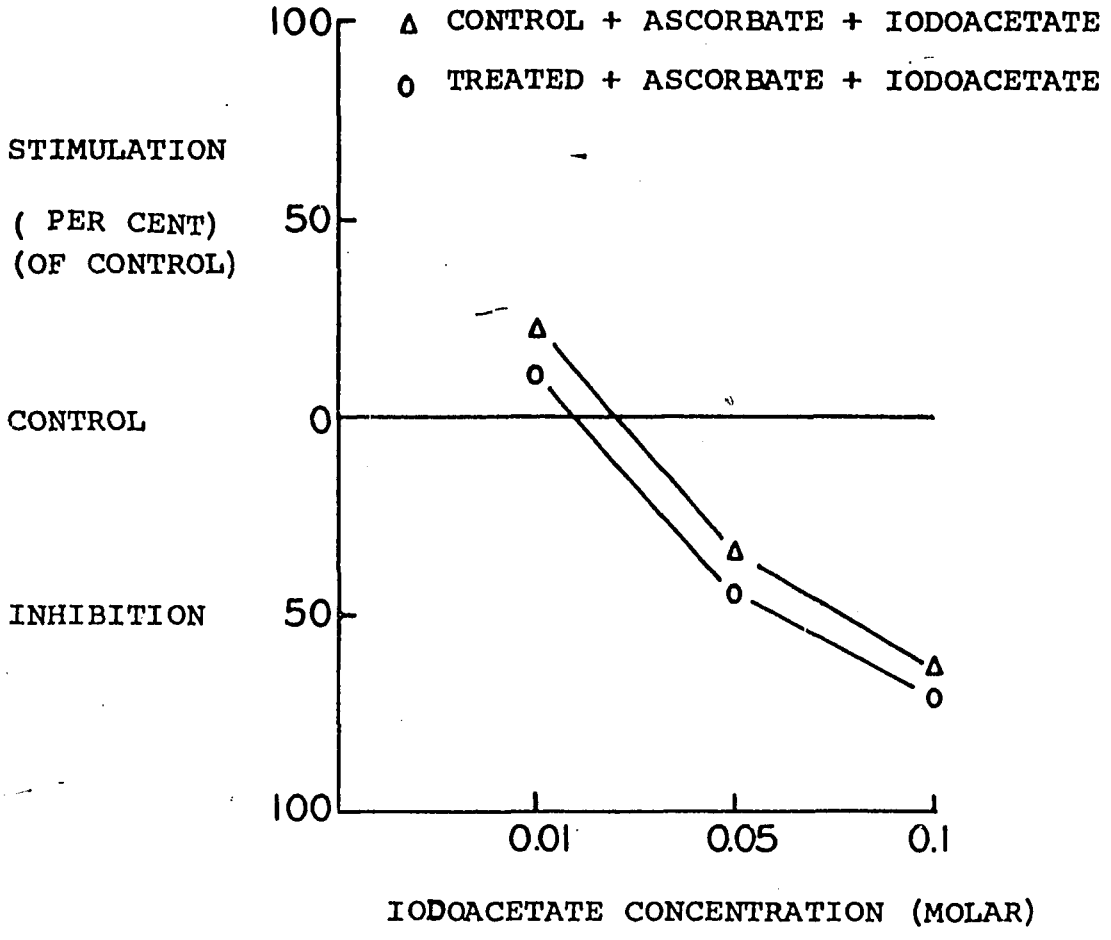


Figure 25. The effect of iodoacetate on the oxidation of ascorbic acid by control and dicryl treated corn homogenates.

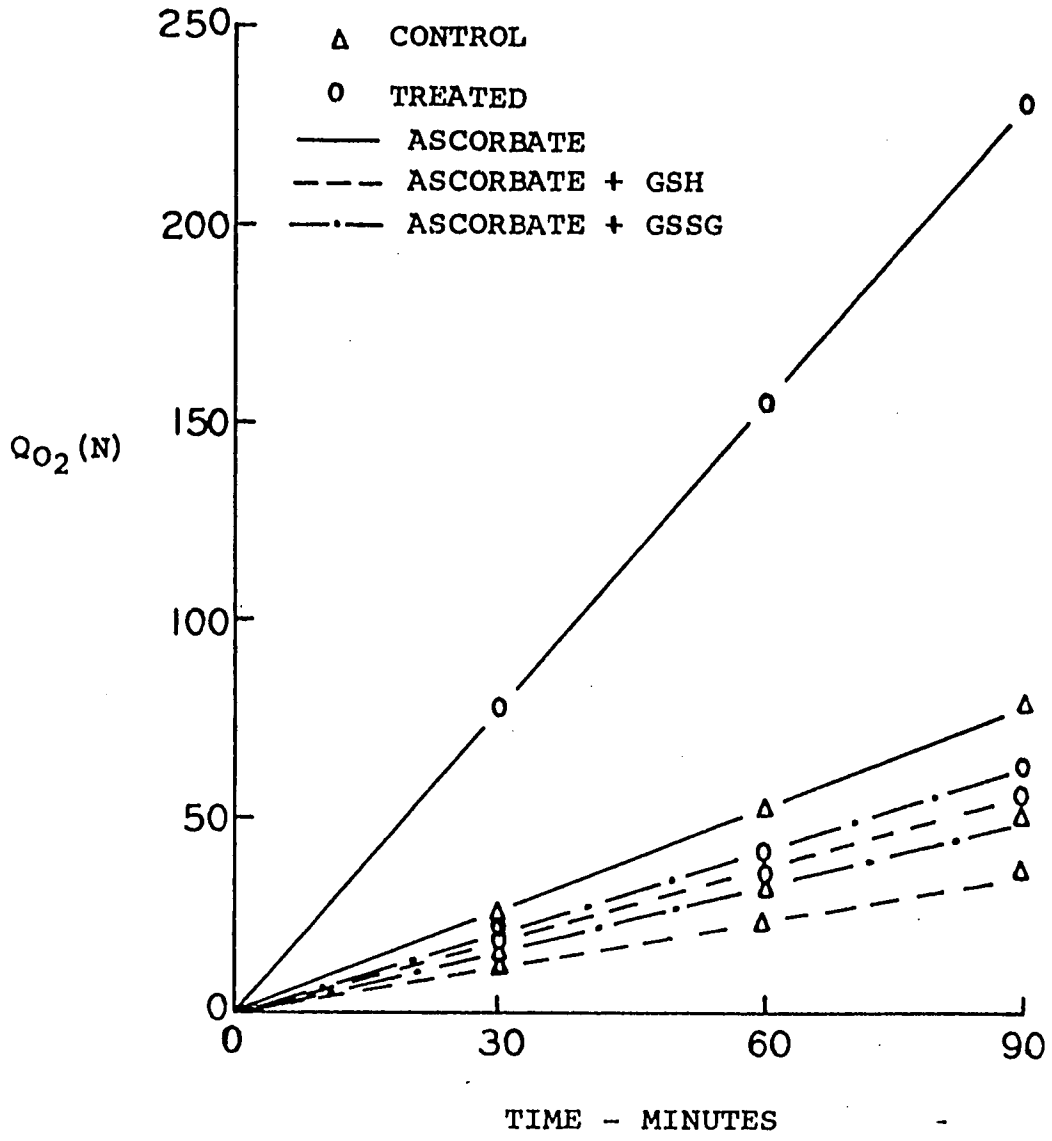


Figure 26. The effect of 0.05 M reduced (GSH) and oxidized (GSSG) glutathione on the oxidation of ascorbic acid by homogenates of control and dicryl treated corn tissue.

(Figure 26). It was concluded if it were possible for the -SH groups to cause any increase in oxygen uptake it was overidden by the proteinaceous nature of the molecule which is known to cause inhibition of oxidation of ascorbate.

M. Fractionation by Column Chromatography

Control and treated supernatants (1,000 xg) from homogenates were fractionated by the use of a column of celite. Eight 10 ml fractions, which were eluted from the column with potassium phosphate buffer, were checked for the oxidation of ascorbate by the usual manometric technique. As the fractions were collected it was noticed that numbers 4, 5, and 6 were of a brownish color, while the remainder were clear. The treated were darker than control. When fractions were checked for the oxidation of ascorbate, it was found that the ones which contained the brownish color also contained the greatest ability to oxidize ascorbate (Figures 27 and 28). The wide differences in treated and control fractions was of the same order of magnitude as it was in homogenates.

After the fractions were collected from the column they were evaporated by two methods and brought back to volume with buffer prior to being checked for the oxidation of ascorbate. One of these methods involved evaporating the fractions under vacuum (Figure 27) and the other involved placing the tubes which contained the fractions in a container of water and boiling them for 2 hours (Figure 28). It is evident that prolonged boiling greatly decreased the activity of the fractions. It could be concluded from this that the substance or substances responsible for oxidation of ascorbate are susceptible to prolonged periods of heat.

The effect of 10 mM KCN on the oxidation of ascorbate by these various fractions was also determined. This heavy metal complexer

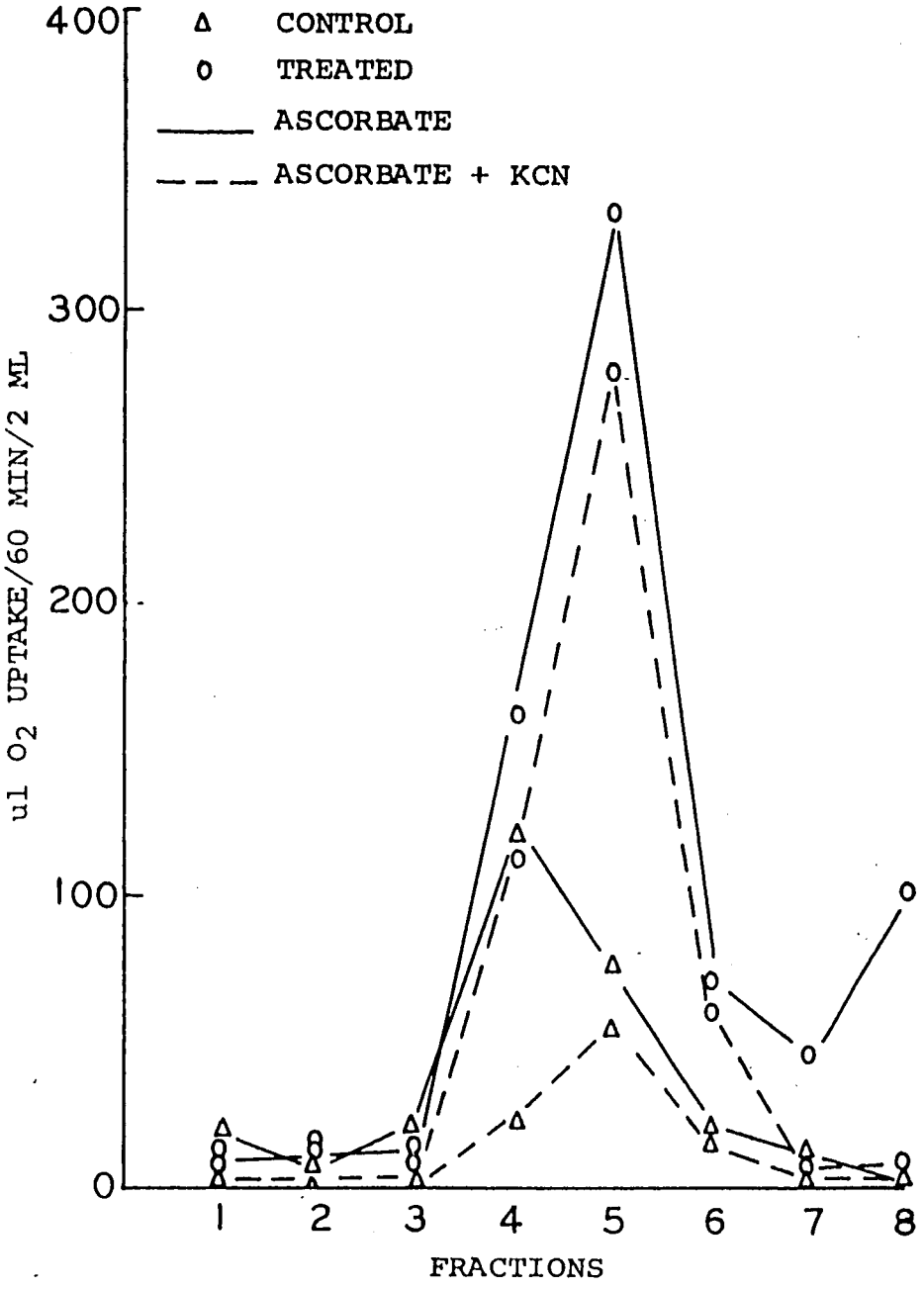


Figure 27. The oxidation of ascorbic acid by fractions from control and treated tissue which were collected from a celite column (fractions were evaporated under vacuum). Potassium cyanide was added at a concentration of 10 mM.

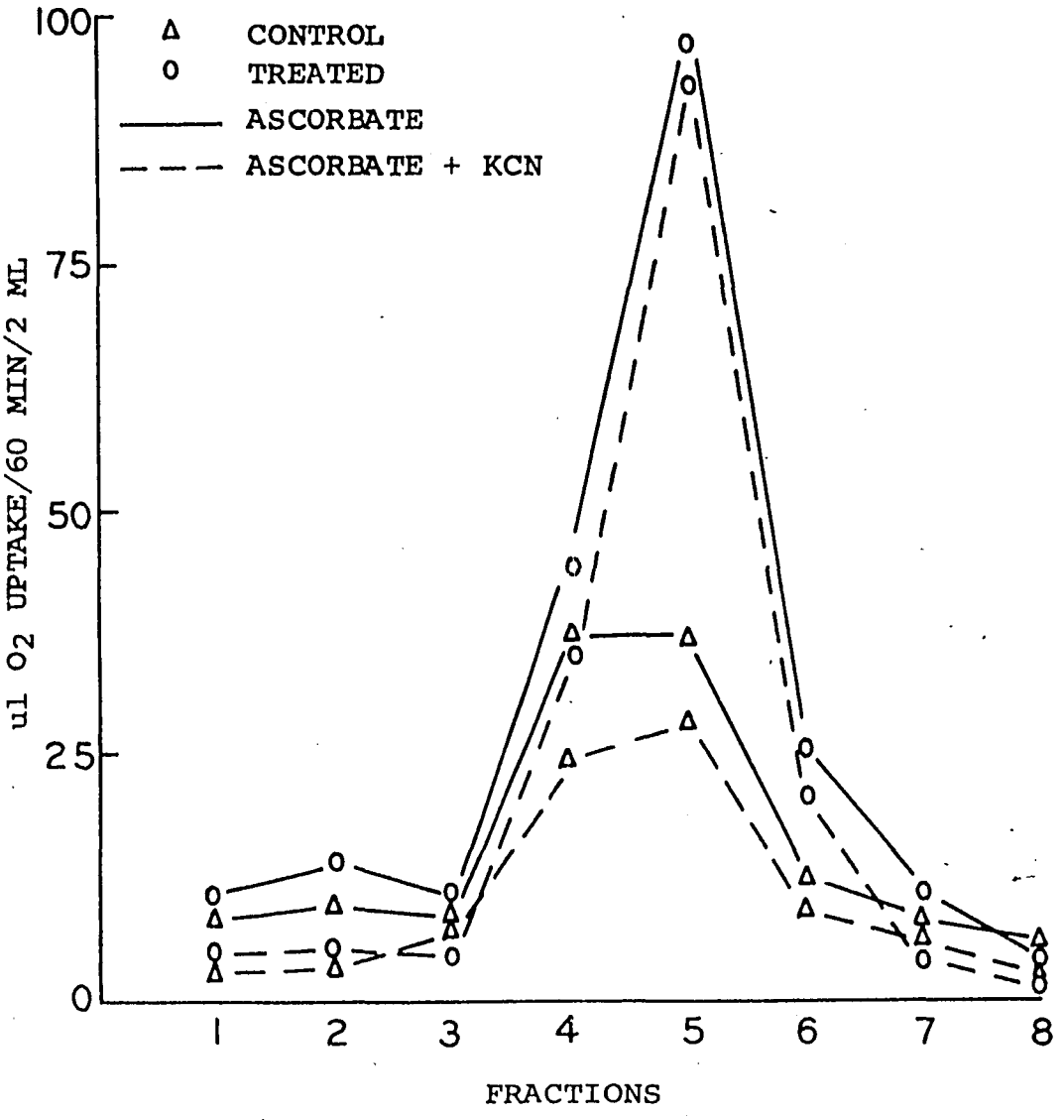


Figure 28. The oxidation of ascorbic acid by fractions from control and treated tissue which were collected from a celite column (fractions were evaporated by boiling). Potassium cyanide was added at a concentration of 10 mM.

caused only a very slight inhibition of the oxidation of ascorbate in all fractions (Figures 27 and 28).

Separation of the brown color from ascorbate activity appeared to be the next step that should be pursued in the investigation. This was done by the use of a 98 per cent celite and 2 per cent activated charcoal column. After supernatants were placed on the column, ten 10 ml fractions were eluted with buffer and then 10 additional fractions were eluted with a 10 per cent pyridine solution. Ascorbate activity came off the column in fractions 3, 4, and 5, and these fractions were clear (Figure 29). The brownish color appeared in fraction 14 and 15 which were eluted with 10 per cent pyridine.

The next experiment involved the effect of $CuSO_4$ on the oxidation of ascorbate by the above fractions. Some of the data collected in this experiment are illustrated in Figure 30. This figure contains data from treated tissue only as data collected from control tissue was very similar. It was found that copper catalyzed oxidation of ascorbate was greatly influenced by the various fractions. It can be seen that the rate of oxidation of ascorbate followed the non-copper catalyzed oxidation very closely at fractions 3, 4, and 5 (Figure 30). The rate of oxidation of other fractions, however, remained close to the check (buffer, $CuSO_4$, ascorbate) with the exception of fractions 14 and 15. It should be re-emphasized that fractions 14 and 15 contained a brownish color and did not cause any appreciable oxidation of ascorbate prior to the addition of copper.

A 0.04 ml aliquot from each of the 20 fractions was placed on chromatographic paper. This was dried immediately and sprayed with ninhydrin. After the paper was dried again it could be seen that fractions 4, 5, 14 and 15 exhibited considerably more color than any of the

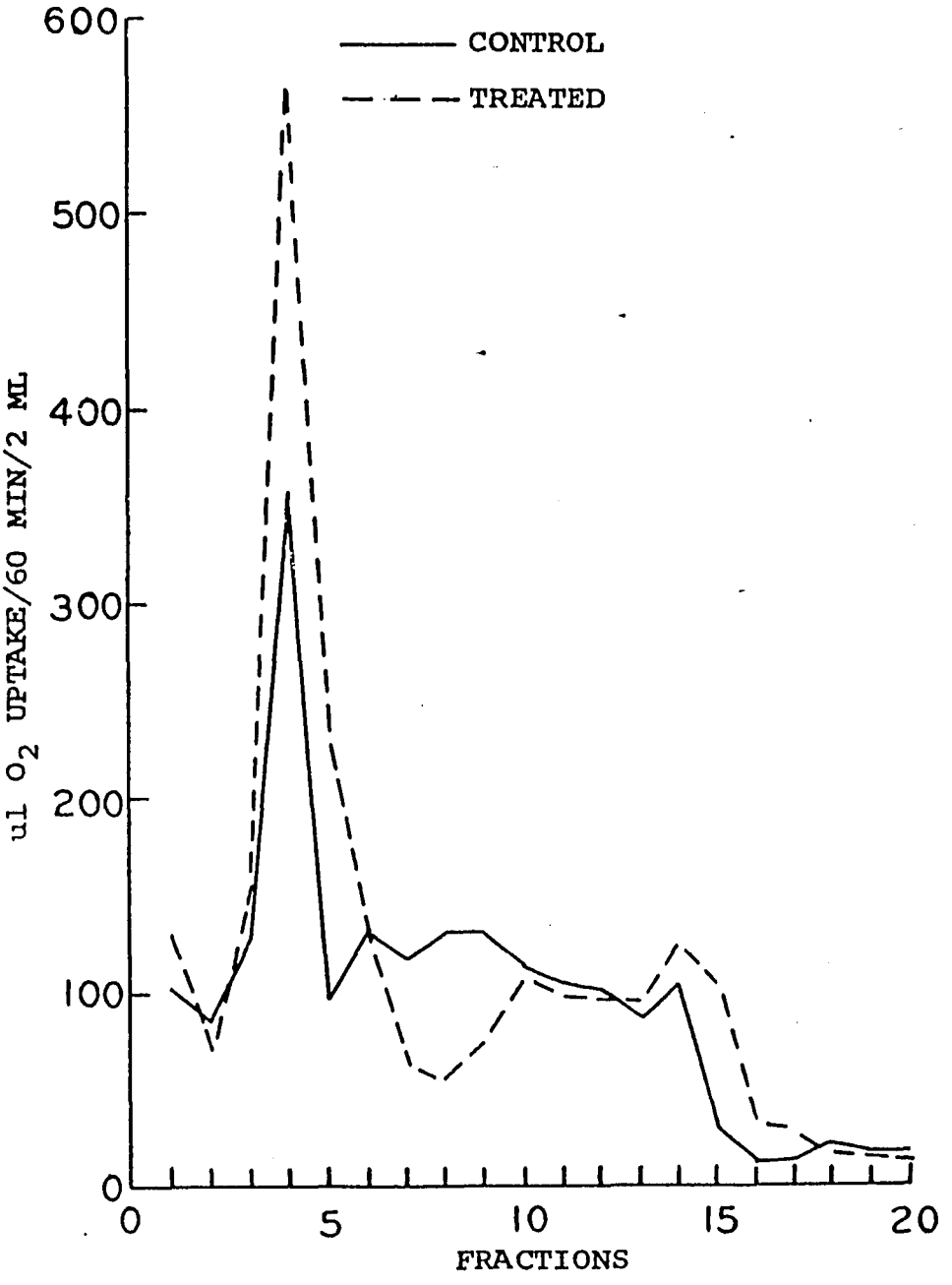


Figure 29. Ascorbic acid oxidation by fractions from control and treated tissue which were collected from a celite (98 per cent) - charcoal (2 per cent) column. Fractions 1-10 were eluted with buffer and fractions 11-20 were eluted with 10 per cent pyridine.

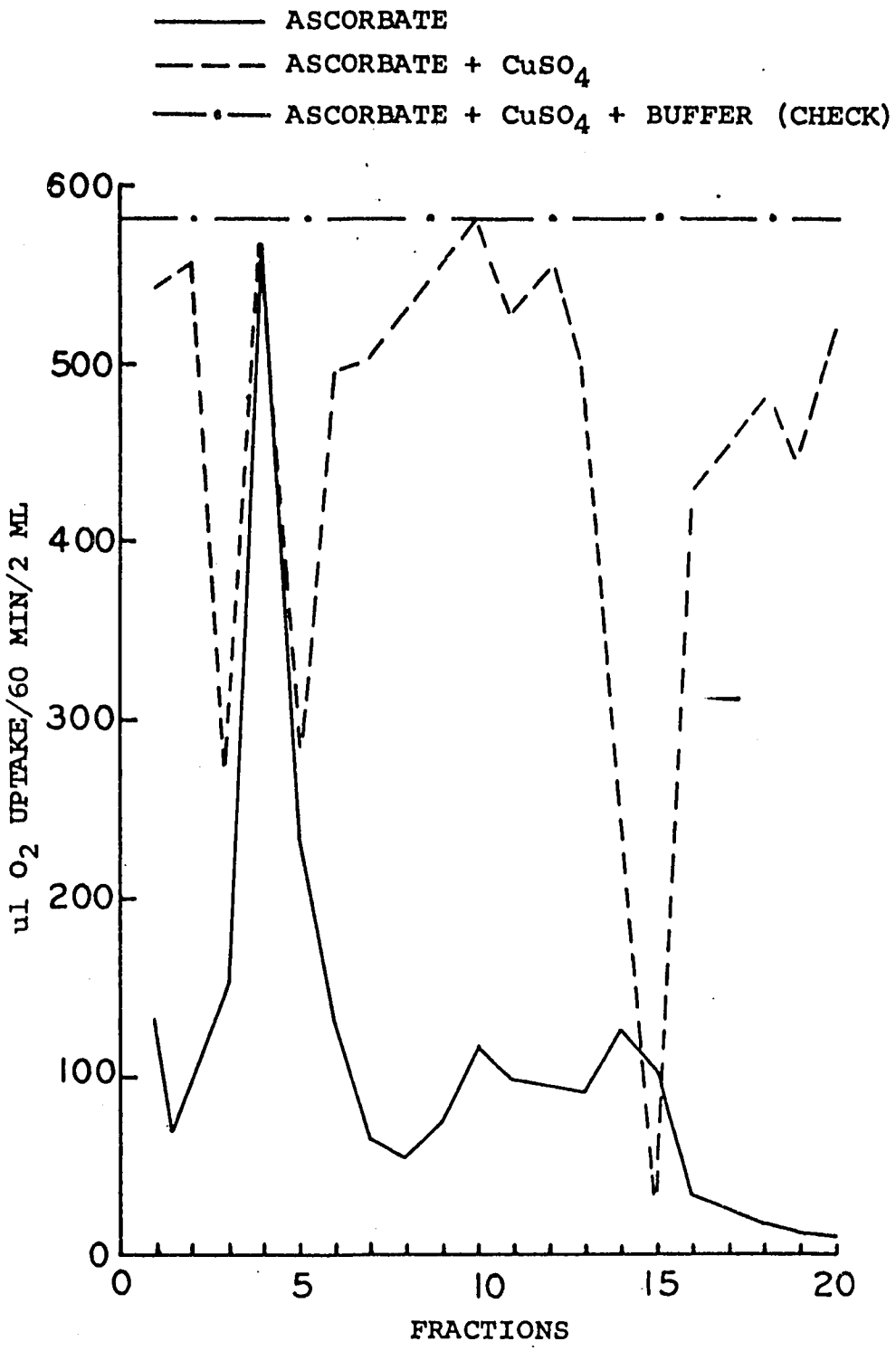


Figure 30. The effect of copper (1ppm $CuSO_4$) on the oxidation of ascorbate by several fractions which were collected from a 98 per cent celite - 2 per cent charcoal column (treated tissue only).

fractions which followed fractions 5 and 16.

It was concluded that the inhibition of ascorbate in these fractions was due to the presence of amino acids. This type of inhibition has been experienced previously (Figures 24 and 26). The substance or substances responsible for the oxidation of ascorbate are still unknown. This investigation, however, presents evidence that the reaction is of a nonenzymatic nature.

SUMMARY

Some of the effects of N-(3,4-dichlorophenyl) methacrylamide (dicryl) on corn were investigated.

Dicryl caused a slight reduction in length of corn coleoptiles while IAA caused a significant increase in elongation. Dicryl had no influence on the elongation of coleoptiles which were also treated with IAA.

When dicryl was applied to the roots of corn it was found that the dry weight of both roots and shoots was reduced.

Dicryl caused a sudden decrease in the respiration of corn leaf tissue. The rate of respiration of control tissue steadily decreased during the testing period. The effect of isophorone was intermediate between control and dicryl treated tissue.

Sodium fluoride, iodoacetate, sodium azide, and malonic acid all caused some decrease in the respiration of control and treated tissues. There did not appear to be any significant difference in the effect of any one of these chemicals on treated and control tissue. Respiration was stimulated by low concentrations of 2,4-DNP and inhibited by high concentrations. Treated tissue appeared to be more sensitive to DNP than control.

Phenol oxidase was the most active of the terminal oxidases. Negative results were obtained for participation of the cytochrome system in respiration of corn.

Dicryl caused little effect on catalase activity 2 days after treatment but 6 days after treatment it caused a 50 per cent reduction

in activity. The effect of the herbicide on peroxidase and glycolic acid oxidase was essentially the same as that encountered with catalase.

Three to 4 days after corn was treated with dicryl there was a sudden increase in the oxidation of ascorbate which continued to rise throughout the testing period. The oxidation of ascorbate by control and treated tissue was slightly increased by boiling the homogenates or supernatants.

The addition of $CuSO_4$ to homogenates or supernatants from control and treated tissue caused a slight increase in the oxidation of ascorbate.

The oxidation of ascorbate by control and treated corn tissue was not influenced by the presence of heavy metal complexers. Iodoacetate, however, caused considerable inhibition at relatively high concentrations.

The batch treatment of supernatants with a cation exchange resin had little or no effect on the oxidation of ascorbate.

The possibility of ascorbate serving as a source of electrons for the reduction of quinones proved to be negative.

Dialysis studies indicated considerably more nitrogen was lost from dialyzed treated than from dialyzed control supernatants; therefore, it was concluded that the treatment with dicryl caused a breakdown of nitrogenous compounds.

More nitrogen was precipitated in control than in treated homogenates which were centrifuged at 1,000 to 144,000 xg. It was found that the same speeds of centrifugation had little effect on the oxidation of ascorbate by the supernatants.

It was found that neither dehydroascorbate nor hydroquinone could substitute for ascorbate.

The oxidation of ascorbate by cucumber extract was inhibited by boiling and by 0.2 mM dieca. There was nothing in corn homogenates which affected the oxidation of ascorbate in cucumber extract and vice versa.

Paper chromatography studies indicated there was considerably more alanine, asparagine, and glutamine in treated than in control tissue. The oxidation of ascorbate by homogenates from control and treated tissue was affected very little when these amino acids were added at low concentrations, but at higher concentrations glutamine and asparagine caused some inhibition.

The effect of reduced (GSH) and oxidized (GSSG) glutathione on the oxidation of ascorbate was the same as that encountered with the higher concentrations of amino acids.

When supernatants (1,000 xg) from homogenates were fractionated into eight 10 ml fractions by the use of a column of celite, it was found that fractions 4 and 5 oxidized ascorbate quite rapidly. These fractions were also of a brownish color. The color was separated from ascorbate activity by chromatography on a 98 per cent celite - 2 per cent charcoal column. Copper increased greatly the oxidation of ascorbate in all fractions from the celite-charcoal column except the ones which exhibited ascorbate activity prior to the addition of copper and the ones which contained the brown color.

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BIOGRAPHY

Henry Hanly Funderburk, Jr. was born in Carrollton, Alabama, June 19, 1931: son of Henry Hanly and Mary Louise (Ferguson) Funderburk. He attended school at Carrollton High School of Carrollton, Alabama, graduating in June, 1949.

He enrolled at Auburn University at Auburn, Alabama, in September, 1949, and received the degree of Bachelor of Science in Agricultural Science and a commission in the U. S. Army in June, 1953.

In October, 1953, he entered the U. S. Army. He served with the First Armored Division and was discharged in October, 1955. In November, 1955, he accepted a position as Assistant in Botany at Auburn University. In January, 1956, he enrolled in the Graduate School for advanced work in the Department of Botany and Plant Pathology and received the Master of Science degree in December, 1958.

He was married on July 26, 1953, to Helen Louise Hanson and has two children, a daughter, Debra Elaine Funderburk, born May 21, 1955, and a son, Kenneth Cliff Funderburk, born February 25, 1959.

He entered Louisiana State University in February, 1959, as a Graduate Research Assistant in the Department of Botany, Bacteriology and Plant Pathology. He is a candidate for the degree of Doctor of Philosophy in January, 1961.

EXAMINATION AND THESIS REPORT

Candidate: Henry Hanly Funderburk, Jr.

Major Field: Botany

Title of Thesis: A Study of Some Physiological Processes of Corn as Affected by N-(3,4-Dichlorophenyl) Methacrylamide

Approved:

Walter K. Porter, Jr.
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Richard J. Russell
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Date of Examination:

December 16, 1960